

**ALPHA (2) MACROGLOBULIN RECEPTOR AS A HEAT SHOCK PROTEIN  
RECEPTOR AND USES THEREOF**

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5 This application is a continuation-in-part of co-pending application number 09/625,137, filed July 25, 2000, which claims the benefit under 35 U.S.C. § 119(e) to provisional application no. 60/209,095, filed June 2, 2000, each of which is incorporated by reference herein in its entirety.

10 The invention was made with government support under grant number CA64394 awarded by the National Institutes of Health. The government has certain rights in the invention.

**1. INTRODUCTION**

15 The present invention relates to the use of alpha (2) macroglobulin ("α2M") receptor as a heat shock protein receptor, cells that express the α2M receptor bound to an HSP, and antibodies and other molecules that bind the α2M receptor-HSP complex. The invention also relates to screening assays to identify compounds that modulate the interaction of an HSP with the α2M receptor, and methods for using compositions comprising α2M-receptor  
20 sequences for the diagnosis and treatment of immune disorders, proliferative disorders, and infectious diseases.

**2. BACKGROUND OF THE INVENTION**

**2.1. HEAT SHOCK PROTEINS**

25 Heat shock proteins (HSPs), also referred to as stress proteins, were first identified as proteins synthesized by cells in response to heat shock. Hsps have classified into five families, based on molecular weight, Hsp100, Hsp90, Hsp70, Hsp60, and smHsp. Many members of these families were found subsequently to be induced in response to other stressful stimuli including nutrient deprivation, metabolic disruption, oxygen radicals, and  
30 infection with intracellular pathogens (see Welch, May 1993, Scientific American 56-64; Young, 1990, Annu. Rev. Immunol. 8:401-420; Craig, 1993, Science 260:1902-1903; Gething *et al.*, 1992, Nature 355:33-45; and Lindquist *et al.*, 1988, Annu. Rev. Genetics 22:631-677).

35 Heat shock proteins are among the most highly conserved proteins in existence. For example, DnaK, the Hsp70 from *E. coli* has about 50% amino acid sequence identity with Hsp70 proteins from excoriates (Bardwell *et al.*, 1984, Proc. Natl. Acad. Sci. 81:848-852).

The Hsp60 and Hsp90 families also show similarly high levels of intra-family conservation (Hickey *et al.*, 1989, Mol. Cell. Biol. 9:2615-2626; Jindal, 1989, Mol. Cell. Biol. 9:2279-2283). In addition, it has been discovered that the Hsp60, Hsp70 and Hsp90 families are composed of proteins that are related to the stress proteins in sequence, for example, having  
5 greater than 35% amino acid identity, but whose expression levels are not altered by stress.

Studies on the cellular response to heat shock and other physiological stresses revealed that the HSPs are involved not only in cellular protection against these adverse conditions, but also in essential biochemical and immunological processes in unstressed cells. HSPs accomplish different kinds of chaperoning functions. For example, members  
10 of the Hsp70 family, located in the cell cytoplasm, nucleus, mitochondria, or endoplasmic reticulum (Lindquist *et al.*, 1988, Ann. Rev. Genetics 22:631-677), are involved in the presentation of antigens to the cells of the immune system, and are also involved in the transfer, folding and assembly of proteins in normal cells. HSPs are capable of binding proteins or peptides, and releasing the bound proteins or peptides in the presence of  
15 adenosine triphosphate (ATP) or low pH.

## 2.2. IMMUNOGENICITY OF HSP-PEPTIDE COMPLEXES

Srivastava *et al.* demonstrated immune response to methylcholanthrene-induced sarcomas of inbred mice (1988, Immunol. Today 9:78-83). In these studies, it was found  
20 that the molecules responsible for the individually distinct immunogenicity of these tumors were glycoproteins of 96kDa (gp96) and intracellular proteins of 84 to 86kDa (Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3407-3411; Ullrich *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3121-3125). Immunization of mice with gp96 or p84/86 isolated from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically  
25 distinct tumors. Isolation and characterization of genes encoding gp96 and p84/86 revealed significant homology between them, and showed that gp96 and p84/86 were, respectively, the endoplasmic reticular and cytosolic counterparts of the same heat shock proteins (Srivastava *et al.*, 1988, Immunogenetics 28:205-207; Srivastava *et al.*, 1991, Curr. Top. Microbiol. Immunol. 167:109-123). Further, Hsp70 was shown to elicit immunity to the  
30 tumor from which it was isolated but not to antigenically distinct tumors. However, Hsp70 depleted of peptides was found to lose its immunogenic activity (Udono and Srivastava, 1993, J. Exp. Med. 178:1391-1396). These observations suggested that the heat shock proteins are not immunogenic per se, but form noncovalent complexes with antigenic peptides, and the complexes can elicit specific immunity to the antigenic peptides  
35 (Srivastava, 1993, Adv. Cancer Res. 62:153-177; Udono *et al.*, 1994, J. Immunol., 152:5398-5403; Suto *et al.*, 1995, Science, 269:1585-1588).

Noncovalent complexes of HSPs and peptide, purified from cancer cells, can be used for the treatment and prevention of cancer and have been described in PCT publications WO 96/10411, dated April 11, 1996, and WO 97/10001, dated March 20, 1997 (U.S. Patent No. 5,750,119 issued April 12, 1998, and U.S. Patent No. 5,837,251 issued November 17, 1998, respectively, each of which is incorporated by reference herein in its entirety). The isolation and purification of stress protein-peptide complexes has been described, for example, from pathogen-infected cells, and can be used for the treatment and prevention of infection caused by the pathogen, such as viruses, and other intracellular pathogens, including bacteria, protozoa, fungi and parasites (see, for example, PCT Publication WO 95/24923, dated September 21, 1995). Immunogenic stress protein-peptide complexes can also be prepared by in vitro complexing of stress protein and antigenic peptides, and the uses of such complexes for the treatment and prevention of cancer and infectious diseases has been described in PCT publication WO 97/10000, dated March 20, 1997 (U.S. Patent No. 6,030,618 issued February 29, 2000). The use of stress protein-peptide complexes for sensitizing antigen presenting cells in vitro for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997 (see also U.S. Patent No. 5,985,270 issued November 16, 1999).

### 2.3. ALPHA (2) MACROGLOBULIN RECEPTOR

The alpha (2) macroglobulin receptor (herein referred to interchangeably as either “ $\alpha$ 2MR” or “the  $\alpha$ 2M receptor”), also known as LDL (low-density lipoprotein) receptor-Related Protein (“LRP”) or CD91, is primarily expressed in liver, brain and placenta. The  $\alpha$ 2M receptor is a member of the low density lipoprotein receptor family. The extracellular domain of the human receptor comprises six 50-amino acid EGF repeats and 31 complement repeats of approximately 40-42 amino acids. The complement repeats are organized, from the amino to the carboxy-terminus, into clusters of 2, 8, 10 and 11 repeats, called Cluster I, II, III and IV (Herz *et al.*, 1988, EMBO J. 7:4119-4127). One study points to Cluster II (C1-II), which contains complement repeats 3-10 (CR3-10), as the major ligand binding portion of the receptor (Horn *et al.*, 1997, J. Biol. Chem. 272:13608-13613). The  $\alpha$ 2M receptor plays a role in endocytosis of a diversity of ligands. In addition to  $\alpha$ 2M, other ligands of  $\alpha$ 2MR include lipoprotein complexes, lactoferrin, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and exotoxins. Thus, the  $\alpha$ 2M receptor plays roles in a variety of cellular processes, including endocytosis, antigen presentation, cholesterol regulation, ApoE-containing lipoprotein clearance, and chylomicron remnant removal.

Human  $\alpha$ 2M is synthesized as a 1474 amino acid precursor, the first 23 of which function as a signal sequence that is cleaved to yield a 1451 amino acid mature protein (Kan et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:2282-2286). In experiments with recombinant protein, the carboxy-terminal 138 amino acids of  $\alpha$ 2M (representing amino acids 1314-1451 of the mature protein) was found to bind the receptor. This domain has been called the RBD (receptor-binding domain; Salvesen *et al.*, 1992, FEBS Lett. 313:198-202; Holtet *et al.*, 1994, FEBS Lett. 344:242-246). An RBD variant (RBDv), a proteolytic fragment of  $\alpha$ 2M comprising an additional 15 amino terminal residues (representing amino acids 1314-1451 of the mature protein) binds to the receptor with almost the same affinity as  $\alpha$ 2M-proteinase

Alignment of  $\alpha$ 2MR ligands identifies a conserved domain present in the RBDs of  $\alpha$  macroglobulins. The conserved sequence spans amino acids 1366-1392 of human  $\alpha$ 2M. Conserved residues within this domain are Phe<sub>1366</sub>, Leu<sub>1369</sub>, Lys<sub>1370</sub>, Val<sub>1373</sub>, Lys<sub>1374</sub>, Glu<sub>1377</sub>, Val<sub>1382</sub>, Arg<sub>1384</sub> (Nielsen *et al.*, 1996, J. Biol. Chem. 271:12909-12912). Of these, Lys<sub>1370</sub> and Lys<sub>1374</sub> were shown to be critical for receptor binding (Nielsen *et al.*, 1996, J. Biol. Chem. 271:12909-12912).

Binding of ligands, including the binding to  $\alpha$ 2M, to  $\alpha$ 2MR is inhibited by  $\alpha$ 2MR-associated protein (RAP). RAP is a 39 kDa folding chaperone that resides in the endoplasmic reticulum and is required for the normal processing of  $\alpha$ 2MR. RAP has the ability to competitively inhibit the binding of all  $\alpha$ 2MR to all  $\alpha$ 2MR ligands tested. One study shows RAP to bind to complement repeats C5-C7 in cluster II (C1-II) of  $\alpha$ 2MR (Horn et al., 1997, J. Biol. Chem. 272:13608-13613); another shows RAP to bind to all two complement repeat-modules in C1-II except the C9-C10 module (Andersen *et al.*, J. Biol. Chem., Mar. 24, 2000, PMID: 10747921; published electronically ahead of print). Three structural domains, 1, 2 and 3, have been identified in RAP, consisting of amino acid residues 18-112, 113-218 and 219-323, respectively. Ligand competition titration of recombinant RAP domains indicates that determinants for the inhibition of test ligands reside in the C-terminal regions of domains 1 and 3 (Ellgaard *et al.*, 1997, Eur. J. Biochem. 244:544-51).

## 2.4. ANTIGEN PRESENTATION

Major histocompatibility complex (MHC) molecules present antigens on the cell surface of antigen-presenting cells. Cytotoxic T lymphocytes (CTLs) then recognize MHC molecules and their associated peptides and kill the target cell. Antigens are processed by two distinct antigen processing routes depending upon whether their origin is intracellular or extracellular. Intracellular or endogenous protein antigens, *i.e.*, antigens synthesized within the antigen-presenting cell, are presented by MHC class I (MHC I) molecules to CD8+



cytotoxic T lymphocytes. On the other hand, extracellular or exogenously synthesized antigenic determinants are presented on the cell surface of "specialized" or "professional" APCs (macrophages, for example) by MHC class II molecules to CD4+ T cells (see, generally, Fundamental Immunology, W.E. Paul (ed.), New York: Raven Press, 1984). This compartmental segregation of antigen processing routes is important to prevent tissue destruction that could otherwise occur during an immune response as a result of shedding of neighboring cell MHC I antigens.

The heat shock protein gp96 chaperones a wide array of peptides, depending upon the source from which gp96 is isolated (for review, see Srivastava *et al.*, 1998, Immunity 8: 657-665). Tumor-derived gp96 carries tumor-antigenic peptides (Ishii *et al.*, 1999, J. Immunology 162:1303-1309); gp96 preparations from virus-infected cells carry viral epitopes (Suto and Srivastava, 1995, Science 269:1585-1588; Nieland *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95:1800-1805), and gp96 preparations from cells transfected with model antigens such as ovalbumin or  $\beta$ -galactosidase are associated with the corresponding epitopes (Arnold *et al.*, 1995, J. Exp. Med. 182:885-889; Breloer *et al.*, 1998, Eur. J. Immunol. 28:1016-1021). The association of gp96 with peptides occurs in vivo (Menoret and Srivastava, 1999, Biochem. Biophys. Research Commun. 262:813-818). Gp96-peptide complexes, whether isolated from cells (Tamura *et al.*, 1997, Science 278:117-120), or reconstituted *in vitro* (Blachere *et al.*, 1997, J. Exp. Med. 186:1183-1406) are excellent immunogens and have been used extensively to elicit CD8+ T cell responses specific for the gp96-chaperoned antigenic peptides.

The capacity of gp96-peptide complexes to elicit an immune response is dependent upon the transfer of the peptide to MHC class I molecules of antigen-presenting cells (Suto and Srivastava, 1995, *supra*). Endogenously synthesized antigens chaperoned by gp96 in the endoplasmic reticulum [ER] can prime antigen-specific CD8+ T cells (or MHC I-restricted CTLs) *in vivo*; this priming of CD8+ T cells requires macrophages. However, the process whereby exogenously introduced gp96-peptide complexes elicit the antigen-specific CD8+ T cell response is not completely understood since there is no established pathway for the translocation of extracellular antigens into the class I presentation machinery. Yet antigenic peptides of extracellular origin associated with HSPs are somehow salvaged by macrophages, channeled into the endogenous pathway, and presented by MHC I molecules to be recognized by CD8+ lymphocytes (Suto and Srivastava, 1995, *supra*; Blachere *et al.*, 1997, J. Exp. Med. 186:1315-22).

Several models have been proposed to explain the delivery of extracellular peptides for antigen presentation. One proposal, known as the "direct transfer" model, suggests that HSP-chaperoned peptides are transferred to MHC I molecules on the cell surface of macrophages for presentation to CD8+ T lymphocytes. Another suggestion is that soluble

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extracellular proteins can be trafficked to the cytosol via constitutive macropinocytosis in bone marrow-derived macrophages and dendritic cells (Norbury *et al.*, 1997, Eur. J. Immunol. 27:280-288). Yet another proposed mechanism is that HSPs are taken up by the MHC class I molecules of the macrophage, which stimulate the appropriate T cells  
5 (Srivastava *et al.*, 1994, Immunogenetics 39:93-98. Others have suggested that a novel intracellular trafficking pathway may be involved for the transport of peptides from the extracellular medium into the lumen of ER (Day *et al.*, 1997, Proc. Natl. Acad. Sci. 94:8064-8069; Nicchitta, 1998, Curr. Opin. in Immunol. 10:103-109). Further suggestions include the involvement of phagocytes which (a) possess an ill-defined pathway to shunt protein  
10 from the phagosome into the cytosol where it would enter the normal class I pathway; (b) digest ingested material in lysosomes and regurgitate peptides for loading on the surface to class I molecules (Bevan, 1995, J. Exp. Med. 182:639-41).

Still others have proposed a receptor-mediated pathway for the delivery of extracellular peptides to the cell surface of APCs for antigen presentation. In view of the  
15 extremely small quantity of gp96-chaperoned antigenic peptides required for immunization (Blachere *et al.*, 1997, *supra*), and the strict dependence of immunogenicity of gp96-peptide complexes on functional antigen presenting cells (APCs) (Udono *et al.*, 1994, Proc. Natl. Acad. Sci. U.S.A. 91:3077-3081), APCs had been proposed to possess receptors for gp96 (Srivastava *et al.*, 1994, Immunogenetics 39:93-98). Preliminary microscopic evidence  
20 consistent with such receptors has been recently obtained (Binder *et al.*, 1998, Cell Stress & Chaperones 3 (Supp.1):2.; Arnold-Schild *et al.*, 1999, J. Immunol. 162: 3757-3760; and Wassenberg *et al.*, 1999, J. Cell Sci. 1:12). One hypothesis is that the mannose receptor is used in the uptake of gp96, but no mechanism has been proposed for the non-glycosylated HSPs, such as Hsp70 (Ciupitu *et al.*, 1998, J. Exp. Med., 187:685-691).

25 The identification and characterization of specific molecules involved in HSP-mediated antigen presentation of peptides could provide useful reagents and techniques for eliciting specific immunity by HSP and HSP-peptide complexes, and for developing novel diagnostic and therapeutic methods.

Citation or discussion of a reference herein shall not be construed as an admission  
30 that such is prior art to the present invention.

### 3. SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for the use of the alpha (2) macroglobulin ("α2M") receptor as a heat shock protein receptor. The invention is based, in  
35 part, on the Applicant's discovery that the α2M receptor is a cell surface receptor for heat shock proteins. In particular, the Applicant has shown that the heat shock protein gp96,

hsp90, hsp70, and calreticulin binds directly to the  $\alpha$ 2M receptor, and that  $\alpha$ 2M inhibits re-  
presentation of gp96, hsp90, hsp70, and calreticulin-chaperoned antigenic peptides by  
macrophages. Because no precedent exists for receptors that recognize abundant and  
intracellular proteins like HSPs, the discovery of an HSP cell surface receptor was highly  
5 unexpected.

The present invention provides compositions comprising complexes of HSPs and the  
 $\alpha$ 2M receptor, and antibodies and other molecules that bind the HSP- $\alpha$ 2M receptor complex.  
The invention also encompasses methods for the use of the  $\alpha$ 2M receptor as a heat shock  
protein receptor, including methods for screening for compounds that modulate the  
10 interaction of HSP and the  $\alpha$ 2M receptor, and methods for treatment and detection of HSP-  
 $\alpha$ 2M receptor-mediated processes and HSP- $\alpha$ 2M receptor-related disorders and conditions,  
such as autoimmune disorders, proliferative disorders and infectious diseases.

The invention provides a method for identifying a compound that modulates an HSP-  
 $\alpha$ 2M receptor-mediated process, comprising: (a) contacting a test compound with a heat  
15 shock protein and an alpha (2) macroglobulin receptor; and (b) measuring the level of alpha  
(2) macroglobulin receptor activity or expression, such that if the level of activity or  
expression measured in (b) differs from the level of alpha (2) macroglobulin receptor activity  
in the absence of the test compound, then a compound that modulates an HSP- $\alpha$ 2M  
receptor-mediated process is identified. In one embodiment of this method the compound  
20 identified is an antagonist which interferes with the interaction of the heat shock protein with  
the alpha (2) macroglobulin receptor, further comprising the step of: (c) determining  
whether the level interferes with the interaction of the heat shock protein and the alpha(2)  
macroglobulin receptor. In another embodiment, the test compound is an antibody specific  
for the alpha (2) macroglobulin receptor. In another embodiment, the test compound is an  
25 antibody specific for alpha (2) macroglobulin. In another embodiment, test compound is an  
antibody specific for a heat shock protein. In another embodiment, the test compound is a  
small molecule. In another yet embodiment, the test compound is a peptide. In another  
embodiment, the peptide comprises at least 5 consecutive amino acids of the alpha (2)  
macroglobulin receptor. In yet another embodiment, the peptide comprises at least 5  
30 consecutive amino acids of alpha (2) macroglobulin. In yet another embodiment, the peptide  
comprises at least 5 consecutive amino acids of a heat shock protein sequence. In another  
embodiment, the compound is an agonist which enhances the interaction of the heat shock  
protein with the alpha (2) macroglobulin receptor. In another embodiment, which the HSP-  
 $\alpha$ 2M receptor-mediated process affects an autoimmune disorder, a disease or disorder  
35 involving disruption of antigen presentation or endocytosis, a disease or disorder involving  
cytokine clearance or inflammation, a proliferative disorder, a viral disorder or other  
infectious disease, hypercholesterolemia, Alzheimer's disease, diabetes, or osteoporosis.

The invention also provides a method for identifying a compound that modulates an HSP- $\alpha$ 2M receptor-mediated process, comprising: (a) contacting a test compound with a heat shock protein and an alpha (2) macroglobulin receptor-expressing cell; and (b) measuring the level of alpha (2) macroglobulin receptor activity or expression in the cell, such that if the level of activity or expression measured in (b) differs from the level of alpha (2) macroglobulin receptor activity in the absence of the test compound, then a compound that modulates an HSP- $\alpha$ 2M receptor-mediated process is identified. In yet another embodiment, wherein the alpha (2) macroglobulin receptor activity measured is the ability to interact with a heat shock protein.

The invention also encompasses a method for identifying a compound that modulates the binding of a heat shock protein to the  $\alpha 2M$  receptor, comprising: (a) contacting a heat shock protein with an alpha (2) macroglobulin receptor, or fragment, or analog, derivative or mimetic thereof, in the presence of a test compound; and (b) measuring the amount of heat shock protein bound to the alpha (2) macroglobulin receptor, or fragment, analog, derivative or mimetic thereof, such that if the amount of bound heat shock protein measured in (b) differs from the amount of bound heat shock protein measured in the absence of the test compound, then a compound that modulates the binding of an HSP to the  $\alpha 2M$  receptor is identified. In another embodiment, alpha (2) macroglobulin receptor contacted in step (a) is on a cell surface. In another embodiment, the alpha (2) macroglobulin receptor is immobilized to a solid surface. In another embodiment, the solid surface is a microtiter dish. In another embodiment, the amount of bound heat shock protein is measured by contacting the cell with a heat shock protein-specific antibody. In yet another embodiment, the heat shock protein is labeled and the amount of bound heat shock protein is measured by detecting the label. In another embodiment, the heat shock protein is labeled with a fluorescent label.

The invention further provides a method for identifying a compound that modulates heat shock protein-mediated antigen presentation by alpha (2) macroglobulin receptor-expressing cells comprising: (a) adding a test compound to a mixture of alpha (2) macroglobulin receptor-expressing cells and a complex consisting essentially of a heat shock protein noncovalently associated with an antigenic molecule, under conditions conducive to alpha (2) macroglobulin receptor-mediated endocytosis; (b) measuring the level of antigen-specific stimulation of cytotoxic T cells by alpha (2) macroglobulin receptor-expressing cells, such that if the level measured in (b) differs from the level of said stimulation in the absence of the test compound, then a compound that modulates heat shock protein-mediated antigen presentation by alpha (2) macroglobulin receptor-expressing cells is identified. In one embodiment of this method, the step of measuring the level of the antigenic molecule presented on the cell surface of step (b) comprises: (i) adding the alpha (2) macroglobulin

receptor-expressing cells formed in step (a) to T cells under conditions conducive to the activation of the T cells; and (ii) comparing the level of activation of said cytotoxic T cells with the level of activation of T cells by an alpha (2) macroglobulin receptor-expressing cell formed in the absence of the test compound, wherein an increase or decrease in level of T cell activation indicates that a compound that modulates heat shock protein-mediated antigen presentation by alpha (2) macroglobulin receptor-expressing cells is identified.

In various embodiments, the heat shock protein used in the methods of the invention is gp96. Alternatively, the heat shock proteins hsp90, hsp70, or calreticulin may be used in various embodiments of the invention.

In another embodiment, the invention provides a method for detecting a heat shock protein-alpha (2) macroglobulin receptor-related disorder in a mammal comprising measuring the level of an HSP-alpha (2) macroglobulin receptor-mediated process in a patient sample, such that if the measured level differs from the level found in clinically normal individuals, then a heat shock protein-alpha (2) macroglobulin receptor-related disorder is detected.

The invention also encompasses kits comprising compositions of the invention. In one embodiment, a kit is provided, packaged in one or more containers, comprising: (a) a purified heat shock protein, nucleic acid encoding a heat shock protein, or cell expressing a heat shock protein; and (b) an alpha (2) macroglobulin receptor polypeptide, nucleic acid encoding an alpha (2) macroglobulin receptor polypeptide, or cell expressing an alpha (2) macroglobulin receptor polypeptide. In one embodiment, the kit the alpha (2) macroglobulin receptor polypeptide, nucleic acid encoding an alpha (2) macroglobulin receptor polypeptide, or cell expressing an alpha (2) macroglobulin receptor polypeptide is purified. In another embodiment, the kit further comprises instructions for use in treating an autoimmune disorder, an infectious disease, or a proliferative disorder.

The invention also provides a method for modulating an immune response comprising administering to a mammal a purified compound that modulates the interaction of a heat shock protein with the alpha (2) macroglobulin receptor. In one embodiment, the compound is an agonist which enhances the interaction of the heat shock protein and the alpha (2) macroglobulin receptor. In another embodiment of this method the compound is an antagonist that interferes with the interaction between the heat shock protein and the  $\alpha$ 2M receptor.

The invention further provides a method for treating an autoimmune disorder comprising administering to a mammal in need of such treatment a purified compound that interferes with the interaction of a heat shock protein with the alpha (2) macroglobulin receptor. In one embodiment of this method the compound is an antagonist that interferes with the interaction between the heat shock protein and the  $\alpha$ 2M receptor. In one



embodiment, the antagonist is an antibody specific for alpha (2) macroglobulin receptor. In another embodiment, the antagonist is an antibody specific for a heat shock protein. In another embodiment, the antagonist is a small molecule. In another embodiment, the antagonist is a peptide. In another embodiment, the peptide comprises at least 5 consecutive amino acids of alpha (2) macroglobulin receptor. In another embodiment, the peptide comprises at least 5 consecutive amino acids of alpha (2) macroglobulin. In another embodiment, the peptide comprises at least 5 consecutive amino acids of a heat shock protein sequence.

The invention further provides a method for increasing the immunopotency of a cancer cell or an infected cell comprising transforming said cell with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes an alpha (2) macroglobulin receptor polypeptide.

Still further, the invention provides a method for increasing the immunopotency of a cancer cell or an infected cell comprising: (a) transforming said cell with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes an alpha (2) macroglobulin receptor polypeptide, and (b) administering said cell to an individual in need of treatment, so as to obtain an elevated immune response.

The invention also provides a recombinant cancer cell transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes an alpha (2) macroglobulin receptor polypeptide. In one embodiment, the recombinant cell is a human cell.

In yet another embodiment, the invention provides a recombinant infected cell transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes an alpha (2) macroglobulin receptor polypeptide. In one embodiment, the recombinant cell is a human cell.

In another embodiment, the invention provides a method for screening for molecules that specifically bind to an  $\alpha$ 2M receptor comprising the steps of: (a) contacting an  $\alpha$ 2M receptor with one or more test molecules under conditions conducive to binding; and (b) determining whether any of said test molecules specifically bind to the  $\alpha$ 2M receptor. In one embodiment of this method, test molecules are potential immunotherapeutic drugs.

The invention also provides a method for identifying a compound that modulates the binding of an  $\alpha$ 2M receptor ligand to the  $\alpha$ 2M receptor comprising: contacting an  $\alpha$ 2M receptor with an  $\alpha$ 2M receptor ligand, or an  $\alpha$ 2M receptor-binding fragment, analog, derivative, or mimetic thereof, in the presence of one or more test compound; and (b) measuring the amount of  $\alpha$ 2M receptor ligand, or fragment, analog, derivative or mimetic thereof, bound to the  $\alpha$ 2M receptor, such that if the amount of bound  $\alpha$ 2M receptor ligand measured in (b) differs from the amount of bound  $\alpha$ 2M measured in the absence of the test

compound, then a compound that modulates the binding of an  $\alpha$ 2M receptor ligand to the  $\alpha$ 2M receptor is identified.

In another embodiment, a method is provided for identifying a compound that modulates the interaction between the  $\alpha$ 2M receptor and an  $\alpha$ 2M receptor ligand, comprising: (a) contacting an  $\alpha$ 2M receptor with one or more test compounds; and (b) measuring the level of  $\alpha$ 2M receptor activity or expression, such that if the level of activity or expression measured in (b) differs from the level of  $\alpha$ 2M receptor activity in the absence of one or more test compounds, then a compound that modulates the interaction between the  $\alpha$ 2M receptor and an  $\alpha$ 2M receptor ligand is identified. In one embodiment, the  $\alpha$ 2M receptor ligand is  $\alpha$ 2M.

In another embodiment, a method is provided for identifying a compound that modulates antigen presentation by  $\alpha$ 2M receptor-expressing cells comprising: (a) adding one or more test compounds to a mixture of  $\alpha$ 2M receptor-expressing cells and a complex comprising an  $\alpha$ 2M receptor ligand and an antigenic molecule, under conditions conducive to  $\alpha$ 2M receptor-mediated endocytosis; (b) measuring the level of stimulation of antigen-specific cytotoxic T cells by the  $\alpha$ 2M receptor-expressing cells, such that if the level measured in (b) differs from the level of said stimulation in the absence of the one or more test compounds, then a compound that modulates antigen presentation by  $\alpha$ 2M receptor-expressing cells is identified.

In another embodiment, the invention provides a method for modulating an immune response comprising administering to a mammal a purified compound that binds to the  $\alpha$ 2M receptor in an amount effective to modulate an immune response in the mammal.

In yet another embodiment, a method for treating or preventing a disease or disorder is provided comprising administering to a mammal a purified compound that binds to the  $\alpha$ 2M receptor in an amount effective to treat or prevent a disease or disorder in the mammal. In one embodiment, the disease or disorder is cancer or an infectious disease.

In a further embodiment, a method is provided for treating an autoimmune disorder comprising administering to a mammal in need of such treatment a purified compound that binds to the  $\alpha$ 2M receptor in an amount effective to treat an autoimmune disorder in the mammal.

In another aspect of the invention, a method is provided for stimulating an immune response in a patient comprising administering to said patient blood which has been withdrawn from said patient and treated to remove an  $\alpha$ 2M receptor ligand. In a specific embodiment, the method further comprises administering to said patient a heat shock protein or a heat shock protein-antigenic peptide complex. In a specific embodiment, blood is administered to said patient by syringe. In another embodiment, said blood is administered to said patient by an intravenous drip.

In another embodiment, a method is provided for stimulating an immune response in a patient comprising: a) removing a  $\alpha$ 2M receptor ligand from blood withdrawn from said patient; and b) returning at least a portion of the  $\alpha$ 2M receptor ligand-depleted blood to said patient.

5 In another embodiment, a method is provided for stimulating an immune response in a patient comprising: a) withdrawing blood from said patient; b) removing a  $\alpha$ 2M receptor ligand from said blood; and c) returning at least a portion of the  $\alpha$ 2M receptor ligand-depleted blood to said patient. In a specific embodiment, the method further comprises after step (a) and before step (c) the step of adding a heat shock protein or a heat shock protein  
10 antigenic-peptide complex to said blood. In a specific embodiment, said blood is returned to said patient by syringe. In another specific embodiment, said blood is returned to said patient by an intravenous drip. In another specific embodiment, the removing a  $\alpha$ 2M receptor ligand from the blood comprises the step of contacting the blood with a solid phase attached to a  $\alpha$ 2M receptor ligand-binding molecule for a time period and under conditions  
15 sufficient to allow binding of  $\alpha$ 2M receptor ligand to the  $\alpha$ 2M receptor ligand-binding molecule solid phase. In another specific embodiment, the  $\alpha$ 2M receptor ligand-binding molecule is  $\alpha$ 2M receptor, or a fragment thereof. In another embodiment, said  $\alpha$ 2M receptor ligand-binding molecule does not bind a heat shock protein. In another embodiment, the  $\alpha$ 2M receptor ligand-binding molecule is an  $\alpha$ 2M receptor ligand-specific antibody, or a  
20 fragment thereof.

In various embodiments, an apheresis system is used in said removing step. In other embodiments blood is withdrawn manually in said withdrawing step. In various embodiments, said removing step comprises separating the plasma from said blood and treating said plasma to remove said  $\alpha$ 2M receptor ligand.

25 The invention further provides a kit comprising in one or more containers a solid phase chromatography column with a purified  $\alpha$ 2M receptor ligand binding molecule attached thereto, such that withdrawn blood can be run over the column to deplete the blood of a  $\alpha$ 2M receptor ligand. In one embodiment, the  $\alpha$ 2M receptor ligand binding molecule of the kit does not bind heat shock proteins.

30 In various embodiments, the  $\alpha$ 2M receptor ligand is  $\alpha$ 2M, a lipoprotein complex, lactoferrin, tissue-type plasminogen activator, urokinase-type plasminogen activator, or an exotoxin.

The term "HSP- $\alpha$ 2M receptor-mediated process" as used herein refers to a process dependent and/or responsive, either directly or indirectly, to the interaction of HSP with the  
35  $\alpha$ 2M receptor. Such processes include processes that result from an aberrant level of expression, synthesis and/or activity of  $\alpha$ 2M receptor, such as endocytic activities relating to the binding of the various  $\alpha$ 2M ligands, including but not limited to HSP,  $\alpha$ 2M, lipoprotein

complexes, lactoferrin, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and exotoxins. Such processes include, but are not limited to, endocytosis, antigen presentation, cholesterol regulation, apoE-containing lipoprotein clearance, and chylomicron remnant removal.

5       The terms "HSP- $\alpha$ 2M receptor-related disorder" and "HSP- $\alpha$ 2M receptor-related condition", as used herein, refers to a disorder and a condition, respectively, involving a HSP- $\alpha$ 2M receptor interaction. Such disorders and conditions may result, for example, from an aberrant ability of the  $\alpha$ 2M receptor to interact with HSP, perhaps due to aberrant levels of HSP and/or  $\alpha$ 2M receptor expression, synthesis and/or activity relative to levels found in  
10 normal, unaffected, unimpaired individuals, levels found in clinically normal individuals, and/or levels found in a population whose levels represent a baseline, average HSP and/or  $\alpha$ 2M receptor levels. Such disorders include, but are not limited to, autoimmune disorders, diseases and disorders involving disruption of antigen presentation and/or endocytosis, diseases and disorders involving cytokine clearance and/or inflammation, proliferative  
15 disorders, viral disorders and other infectious diseases, hypercholesterolemia, Alzheimer's disease, diabetes, and osteoporosis.

      The term " $\alpha$ 2MR ligand" as used herein, refers to a molecule capable of binding to the  $\alpha$ 2M receptor. Such  $\alpha$ 2MR ligands include as well as known ligands, such as, but not limited to,  $\alpha$ 2M and  $\alpha$ 2M complexes, heat shock proteins and heat shock protein complexes,  
20 lipoprotein complexes, lactoferrin, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and exotoxins. In addition,  $\alpha$ 2MR ligands also include molecules which can readily be identified as  $\alpha$ 2MR ligands using standard binding assays well known in the art. Such  $\alpha$ 2MR ligands are typically endocytosed by cell upon binding to the  $\alpha$ 2M receptor.

25

#### 4. BRIEF DESCRIPTION OF THE FIGURES

**FIG. 1A-C .** Identification of an 80 kDa polypeptide as a putative gp96 receptor. **A.** Confocal microscopy of re-presentation-competent RAW264.7 cells stained with gp96-FITC (left panel) and with albumin-FITC (right panel). **B.** SDS-PAGE analysis of detergent  
30 extracts of plasma membranes from surface biotinylated RAW264.7 (re-presentation-competent) or P815 cells (representation-incompetent) eluted from gp96 or albumin-Sepharose (SA) columns and stained with silver stain (top) or avidin-peroxidase (bottom). **C.** gp96-SASD-I<sup>125</sup> was cross-linked to live peritoneal macrophages (MO) or P815 cells, and the cell lysates examined by SDS-PAGE and autoradiography. Various components were  
35 omitted as controls, as indicated.

**FIG. 2A-B.** Anti-p80 antiserum detects an 80 kDa molecule and inhibits re-presentation of gp96-chaperoned AH1 peptide by macrophage. **A.** Pre-immune and immune sera were used to probe blots of plasma membrane extracts of RAW264.7, peritoneal macrophages (both cell types re-presentation-competent), or P815 cells. **B.** Re-presentation of gp96-chaperoned peptide AH1. Sera were added at the final dilution indicated. The solid cross indicates the level of T cell stimulation when the APCs were pulsed directly with the AH1 peptide. The open cross indicates the corresponding value with unpulsed APCs.

**FIG. 3A-C.** Protein microsequencing of the 80 kDa protein. **A.** Analysis of a single tryptic (GALHIYHQR) peptide by tandem- mass spectrometry. All possible b- and y-ion series together with identified b-ion series (red) and y-ion series (blue) are shown. **B.** Collision-induced dissociation (CID) spectrum of this peptide is shown. **C.** Four identified peptides from the  $\alpha$ 2M receptor, peptide mass, and sequence are shown.

**FIG. 4.**  $\alpha$ 2-Macroglobulin inhibits re-presentation of gp96-chaperoned AH1 peptide by macrophage. The solid cross indicates the level of T cell stimulation when the APCs were pulsed directly with the AH1 peptide. The open cross indicates the corresponding value with unpulsed APCs.

**FIG. 5.** Table of specific binding of HSPs and  $\alpha$ 2-macroglobulin to primary cultures and cell lines of several histological origins. The “\*\*\*” indicates percentage of cells staining with FITC over background staining alone. The “#” indicates that the cells were examined by confocal microscopy. All CD11c<sup>+</sup> cells were intensely positive for binding to the three HSPs and  $\alpha$ 2M..

**FIG. 6A-B.** Analysis of cells by flow cytometry for the presence of FITC labelled cells. The macrophage cell lines RAW264.7 (**A**) or RAW309Cr.1 (**B**) were incubated with 100mg/ml of FITC labeled gp96, hsp90, hsp70 or SA. Live cells only were gated based on FSC.

**FIG. 7A-B.** Re-presentation of gp96-chaperoned peptides by APCs that bind HSPs and  $\alpha$ 2 macroglobulin. The presence of IFN- $\gamma$  (pg/ml) was assayed as a marker for CTL stimulation. (**A**) Peritoneal macrophage or BM-DCs from C57Bl/6 mice (1X10<sup>4</sup>). (**B**) RAW 264.7 or RAW 309Cr.1 macrophage lines were cultured with gp96 (40 mg/ml) by itself or complexed to the AH1-19 peptide and used to stimulate AH1 specific CTLs (1X10<sup>4</sup>).



**FIG. 8.** Peptides chaperoned by hsp90, CRT, hsp70 and gp96 but not serum albumin are re-presented by RAW264.7 cells. The chaperones, uncomplexed or complexed to the AH1-19 peptide were used to pulse RAW264.7 cells which were tested for their ability to stimulate cognate CTLs.

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**FIG. 9A-C.** Gp96, hsp90, hsp70 and calreticulin utilize a common receptor for re-presentation. **(A)** RAW264.7 cells were pulsed with gp96-AH1-19 complexes (40 mg/ml gp96) in presence of increasing concentrations of uncomplexed gp96, hsp90, hsp70 or SA. **(B)** Re-presentation of AH1-19 complexed to gp96, hsp90, hsp70, CRT or albumin was carried out in presence of increasing concentrations of  $\alpha$ 2-macroglobulin. The data is plotted as percentage inhibition of re-presentation. **(C)** Re-presentation of AH1-19 complexed to gp96, hsp90, hsp70 or calreticulin in presence of increasing concentrations of anti-CD91 antibody. The data is plotted as percentage inhibition of re-presentation.

10

**FIG. 10A-C.** Re-presentation of gp96-chaperoned peptides follows the classical endogenous antigen presentation pathway. **(A)** Requirement of proteasomes. Peritoneal macrophage (1X10<sup>6</sup>) were either treated or untreated with lactacystin (100 mM). The cells were labeled with chromium and used as targets against VSV8 specific CTLs. **(B)** Requirement of TAP as measured in vitro. Peritoneal macrophage from TAP<sup>+/+</sup> or TAP<sup>-/-</sup> mice were cultured with gp96 or gp96-VSV19 complex and VSV8 specific CTL line. Culture supernatants were tested for the presence of IFN- $\gamma$  (pg/ml) as a marker for CTL stimulation. **(C)** Requirement of TAP as measured in vivo. Gp96-VSV19 complex was injected intraperitoneally. After 10 days, spleens were removed and cells were cultured in vitro with VSV8. The lymphocyte cultures were tested for their ability to lyse EL4 cells (dotted line) or EL4 cells pulsed with VSV8 peptide (solid line). Each line re-presents one mouse.

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**FIG. 11.**  $\alpha$ 2M receptor is a sensor of necrotic cell death due to its ability to detect extracellular gp96. Conversely, receptors (psR) for phosphatidyl serine (ps) detect apoptotic cell death.

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**FIG. 12A.** The mouse  $\alpha$ 2MR cDNA (SEQ ID NO:1) and predicted open reading frame of murine  $\alpha$ 2MR protein (Genbank accession no. CAA47817). **B.** The murine  $\alpha$ 2M protein (SEQ ID NO:2), with residues identified by microsequencing an 80 kDa, gp96-interacting fragment of the receptor highlighted in bold.

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**FIG. 13A.** The human  $\alpha$ 2M cDNA (SEQ ID NO:3) and predicted open reading frame of  $\alpha$ 2M protein (SEQ ID NO:4)(Genbank accession no. M11313). **B.** The sequence of the

5 mature human  $\alpha$ 2M protein (SEQ ID NO:5), following cleavage of the N-terminal 23 amino acid signal sequence. Highlighted residues represent the 138 amino acid  $\alpha$ 2MR-binding domain (RBD). Underlined residues represent an extension of the RBD that is present in a  $\alpha$ 2MR-binding, proteolytic fragment of  $\alpha$ 2M (RBDv). Bolded residues have been shown to be important for  $\alpha$ 2MR binding. Italicized residues represent a domain that is conserved among ligands of  $\alpha$ 2MR.

10 **FIG. 14A.** The human  $\alpha$ 2MR cDNA (SEQ ID NO:6) and predicted open reading frame of human  $\alpha$ 2MR protein (Genbank accession no. NP\_002323). **B.** Primary amino acid sequence of human  $\alpha$ 2MR (SEQ ID NO:7). The approximate locations of complement repeat clusters I and II are highlighted in grey. Individual complement repeats of C1-II are indicated as follows: amino acids of CR3, 5, 7 and 9 are in italics, and amino acids of CR4, 6, 8, and 10 are underlined. Amino acids highlighted in bold were present in an 80kDa peptide fragment of the mouse  $\alpha$ 2MR that bound to gp96. The double underlined residues represent the predicted signal peptide. For the locations of other features of the receptor, such as the EGF repeats, see the article by (Herz *et al.*, 1988, EMBO J. 7:4119-4127).

## 5. DETAILED DESCRIPTION OF THE INVENTION

20 The present invention relates to compositions and methods for the use of the alpha (2) macroglobulin receptor (also referred to interchangeably herein as " $\alpha$ 2MR" or "the  $\alpha$ 2M receptor") as a heat shock protein ("HSP") receptor. In particular, the present invention provides compositions comprising isolated  $\alpha$ 2MR- ligand complexes, *e.g.*,  $\alpha$ 2MR-HSP complexes, including isolated and/or recombinant cells, and antibodies, molecules and  
25 compounds that modulate the interaction of  $\alpha$ 2MR with an  $\alpha$ 2MR ligand, such as HSP. The invention further encompasses methods for the use of  $\alpha$ 2MR as a heat shock protein receptor, including screening assays to identify compounds that modulate the interaction of  $\alpha$ 2MR with an HSP, or other  $\alpha$ 2MR ligand, and methods for the use of these molecules and complexes for the diagnosis and treatment of immune disorders, proliferative disorders, and  
30 infectious diseases.

The term " $\alpha$ 2MR ligand" as used herein, refers to a molecule capable of binding to the  $\alpha$ 2M receptor. Such  $\alpha$ 2MR ligands include as well as known ligands, such as, but not limited to,  $\alpha$ 2M and  $\alpha$ 2M complexes, heat shock proteins and heat shock protein complexes, lipoprotein complexes, lactoferrin, tissue-type plasminogen activator (tPA), urokinase-type  
35 plasminogen activator (uPA), and exotoxins. In addition,  $\alpha$ 2MR ligands also include molecules which can readily be identified as  $\alpha$ 2MR ligands using standard binding assays

well known in the art. Such  $\alpha$ 2MR ligands are typically endocytosed by cell upon binding to  $\alpha$ 2MR.

An HSP useful in the practice of the invention may be selected from among any cellular protein that satisfies any one of the following criteria: the intracellular concentration of an HSP increases when a cell is exposed to a stressful stimulus; an HSP can bind other proteins or peptides, and can release the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH; or an HSP possesses at least 35% homology with any cellular protein having any of the above properties. Preferably, the HSP used in the compositions and methods of the present invention includes, but are not limited to, HSP90, gp96, BiP, Hsp70, DnaK, Hsc70, PhoE calreticulin, PDI, or an sHsp, alone or in combination.

In a preferred embodiment, an HSP is a mammalian (*e.g.*, mouse, rat, primate, domestic animal such as dog, cat, cow, horse), and is most preferably, human.

Hsps useful in the practice of the invention include, but are not limited to, members of the HSP60 family, HSP70 family, HSP90 family, HSP100 family, sHSP family, calreticulin, PDI, and other proteins in the endoplasmic reticulum that contain thioredoxin-like domain(s), such as, but not limited to, ERp72 and ERp61.

HSP analogs, muteins, derivatives, and fragments can also be used in place of HSPs according to the invention. An HSP peptide-binding "fragment" for use in the invention refers to a polypeptide comprising a HSP peptide-binding domain that is capable of becoming non-covalently associated with a peptide to form a complex that is capable of eliciting an immune response. In one embodiment, an HSP peptide-binding fragment is a polypeptide comprising an HSP peptide-binding domain of approximately 100 to 200 amino acids.

Databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. Such nucleotide sequences of non-limiting examples of HSPs that can be used for preparation of the HSPs used in the methods of the invention are as follows: human Hsp70, Genbank Accession No. NM\_005345, Sargent *et al.*, 1989, Proc. Natl. Acad. Sci. U.S.A., 86:1968-1972; human Hsp90, Genbank Accession No. X15183, Yamazaki *et al.*, Nucl. Acids Res. 17:7108; human gp96: Genbank Accession No. X15187, Maki *et al.*, 1990, Proc. Natl. Acad. Sci., 87: 5658-5562; human BiP: Genbank Accession No. M19645; Ting *et al.*, 1988, DNA 7: 275-286; human Hsp27, Genbank Accession No. M24743; Hickey *et al.*, 1986, Nucleic Acids Res. 14:4127-45; mouse Hsp70: Genbank Accession No. M35021, Hunt *et al.*, 1990, Gene, 87:199-204; mouse gp96: Genbank Accession No. M16370, Srivastava *et al.*, 1987, Proc. Natl. Acad. Sci., 85:3807-3811; and mouse BiP: Genbank Accession No. U16277, Haas *et al.*, 1988,

Proc. Natl. Acad. Sci. U.S.A., 85: 2250-2254. Due to the degeneracy of the genetic code, the term "HSP sequence", as used herein, refers not only to the naturally occurring amino acid and nucleotide sequence but also encompasses all the other degenerate sequences that encode the HSP.

5       The aforementioned HSP families also contain proteins that are related to HSPs in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress. Therefore, it is contemplated that the definition of heat shock or stress protein, as used herein, embraces other proteins, mutants, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to  
10 85% amino acid identity with members of these families whose expression levels in a cell are enhanced in response to a stressful stimulus. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA  
15 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al., 1990, J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein  
20 searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res.25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Altschul  
25 *et al.*, 1997, *supra*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (see <http://www.ncbi.nlm.nih.gov>). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4:11-17. Such an algorithm is incorporated into the ALIGN  
30 program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

      The immunogenic HSP-peptide complexes of the invention may include any complex containing an HSP and a peptide that is capable of inducing an immune response in a  
35 mammal. The peptides are preferably noncovalently associated with the HSP. Preferred complexes may include, but are not limited to, gp96-peptide complexes, HSP90-peptide complexes, HSP70-peptide complexes, HSP60-peptide complexes, HSP100-peptide

complexes, calreticulin-peptide complexes, and sHSP-peptide complexes. For example, the HSP gp96 which is present in the endoplasmic reticulum of eukaryotic cells and is related to the cytoplasmic HSP90's can be used to generate an effective vaccine containing a gp96-peptide complex.

5       The HSPs,  $\alpha$ 2MR, and/or antigenic molecules for use in the invention can be purified from natural sources, chemically synthesized, or recombinantly produced. Although the HSPs may be allogeneic to the patient, in a preferred embodiment, the HSPs are autologous to the patient to whom they are administered.

## 10   5.1   COMPOSITIONS OF THE INVENTION

      The present invention provides compositions that modulate the interaction between  $\alpha$ 2MR and an  $\alpha$ 2MR ligand, such as, for example, an HSP. Such compositions can be used in methods to elicit or modulate an immune response. Such compositions also include antibodies that specifically recognize HSP-  $\alpha$ 2MR complexes, isolated cells that express  
15   HSP- $\alpha$ 2MR complexes, and isolated and recombinant cells that contain recombinant  $\alpha$ 2MR and HSP sequences. In addition, in various methods of the invention, sequences encoding  $\alpha$ 2MR, an HSP, and  $\alpha$ 2M are used for immunotherapy. Such compositions can be used, for example, in immunotherapy against proliferative disorders, infectious diseases, and other HSP- $\alpha$ 2MR-related disorders. Methods for the synthesis and production of such  
20   compositions are described herein.

### 5.1.1   RECOMBINANT EXPRESSION

      In various embodiments of the invention, sequences encoding the  $\alpha$ 2MR, an HSP,  $\alpha$ 2M, or other  $\alpha$ 2MR ligand are inserted into an expression vector for propagation and  
25   expression in recombinant cells. Thus, in one embodiment, the  $\alpha$ 2M receptor, HSP,  $\alpha$ 2M, or other  $\alpha$ 2MR ligand coding region is linked to a non-native promoter for expression in recombinant cells.

      The amino acid sequence of the portion of  $\alpha$ 2MR that recognizes and binds to HSPs is shown in FIG. 12B (SEQ ID NO:2). Based on the discovery by the Applicant, this  
30   portion of  $\alpha$ 2MR is responsible for recognizing and binding to HSPs and HSP-antigenic peptide complexes. After binding HSPs,  $\alpha$ 2MR facilitates transport of the HSP-antigenic peptide complex into the cell, where the peptide antigens associate with MHC class I molecules and are then presented on the cell surface of the cell, and become available to stimulate an immune response. Based on this invention, compositions comprising agonists  
35   and antagonists of  $\alpha$ 2MR and HSPs interactions can be used to modulate the immune response. Thus, recombinant  $\alpha$ 2MR polypeptides, complexes of  $\alpha$ 2MR and an HSP or HSP-



antigenic peptide complexes, and recombinant cells expressing  $\alpha$ 2MR or complexes comprising  $\alpha$ 2MR and antigenic peptides can be used in methods for immunotherapy and diagnostic methods described herein.

In various embodiments of the invention, sequences encoding the  $\alpha$ 2MR, and/or a heat shock protein or  $\alpha$ 2M, or fragments thereof, are inserted into an expression vector for propagation and expression in recombinant cells. An expression construct, as used herein, refers to a nucleotide sequence encoding a particular gene product, such as the  $\alpha$ 2MR, HSP or  $\alpha$ 2M, operably associated with one or more regulatory regions which allows expression of the encoded gene product in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the nucleotide sequence encoding the gene product to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation.

The DNA may be obtained from known sequences derived from sequence databases by standard procedures known in the art by DNA amplification or molecular cloning directly from a tissue, cell culture, or cloned DNA (e.g., a DNA "library"). Any eukaryotic cell may serve as the nucleic acid source for obtaining the coding region of an hsp gene. Nucleic acid sequences encoding HSPs can be isolated from vertebrate, mammalian, as well as primate sources, including humans. Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the hsp gene should be cloned into a suitable vector for propagation of the gene.

Vectors based on *E. coli* are the most popular and versatile systems for high level expression of foreign proteins (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* may include but not limited to *lac*, *trp*, *lpp*, *phoA*, *recA*, *tac*,  $\lambda P_L$ , and phage T3 and T7 promoters (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of prokaryotic expression vectors may include the  $\lambda$ gt vector series such as  $\lambda$ gt11 (Huynh et al., 1984 in "DNA Cloning Techniques", Vol. I: A Practical Approach (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier et al., 1990, Methods Enzymol., 185:60-89). However, a potential drawback of a prokaryotic host-vector system is the inability to perform many of the post-translational processing events of mammalian cells. Thus, an eukaryotic host-vector system is preferred, a mammalian host-vector system is more preferred, and a human host-vector system is the most preferred.

The regulatory regions necessary for transcription of an  $\alpha$ 2MR sequence, for example, can be provided by the expression vector. A translation initiation codon (ATG) may also be provided to express a nucleotide sequence encoding an  $\alpha$ 2M receptor that lacks an initiation codon. In a compatible host-construct system, cellular proteins required for

transcription, such as RNA polymerase and transcription factors, will bind to the regulatory regions on the expression construct to effect transcription of the  $\alpha$ 2MR sequence in the host organism. The precise nature of the regulatory regions needed for gene expression may vary from host cell to host cell. Generally, a promoter is required which is capable of binding  
5 RNA polymerase to initiate the transcription of an operably-associated nucleic acid sequence. Such regulatory regions may include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, the cap site, a CAAT box, and the like. The non-coding region 3' to the coding sequence may contain transcriptional termination regulatory sequences, such as terminators and polyadenylation sites.

10 Both constitutive and inducible regulatory regions may be used for expression of the  $\alpha$ 2M receptor, HSP,  $\alpha$ 2M, or other  $\alpha$ 2MR ligand. It may be desirable to use inducible promoters when the conditions optimal for growth of the recombinant cells and the conditions for high level expression of the gene product are different. Examples of useful regulatory regions are provided in the next section below.

15 For expression of the  $\alpha$ 2M receptor, HSP,  $\alpha$ 2M, or other  $\alpha$ 2MR ligand gene product in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the  
20 metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the  $\beta$ -interferon gene, and the Hsp70 gene (Williams *et al.*, 1989, Cancer Res. 49:2735-42; Taylor *et al.*, 1990, Mol. Cell Biol., 10:165-75). It may be advantageous to use heat shock promoters or stress promoters to drive expression of  $\alpha$ 2MR in recombinant host cells.

25 The following animal regulatory regions, which exhibit tissue specificity and have been utilized in transgenic animals, can also be used in tumor cells of a particular tissue type: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in  
30 pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is  
35 active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is

active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogam et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

The efficiency of expression of the  $\alpha$ 2M receptor in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein,  $\beta$ -actin (see Bittner et al., 1987, Methods in Enzymol. 153:516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1:36-47).

The expression vector may also contain sequences that permit maintenance and replication of the vector in more than one type of host cell, or integration of the vector into the host chromosome. Such sequences may include but are not limited to replication origins, autonomously replicating sequences (ARS), centromere DNA, and telomere DNA. It may also be advantageous to use shuttle vectors that can be replicated and maintained in at least two types of host cells.

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating or identifying host cells that contain DNA encoding an  $\alpha$ 2M receptor. For long term, high yield production of  $\alpha$ 2M receptor, stable expression in mammalian cells is preferred. A number of selection systems may be used for mammalian cells, including, but not limited, to the Herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in *tk*, *hgprt* or *aprt* cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (*dhfr*), which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neomycin phosphotransferase (*neo*), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hygromycin phosphotransferase (*hyg*), which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147). Other selectable markers, such as but not limited to histidinol and Zeocin<sup>TM</sup> can also be used.

In order to insert the DNA sequence encoding  $\alpha$ 2M receptor, HSP,  $\alpha$ 2M, or other  $\alpha$ 2MR ligand into the cloning site of a vector, DNA sequences with regulatory functions, such as promoters, must be attached to DNA sequences encoding the  $\alpha$ 2M receptor, HSP,

α2M, or other α2MR ligand, respectively. To do this, linkers or adapters providing the appropriate compatible restriction sites may be ligated to the ends of cDNA or synthetic DNA encoding an α2M receptor, by techniques well known in the art (Wu et al., 1987, Methods in Enzymol 152:343-349). Cleavage with a restriction enzyme can be followed by  
5 modification to create blunt ends by digesting back or filling in single-stranded DNA termini before ligation. Alternatively, a desired restriction enzyme site can be introduced into a fragment of DNA by amplification of the DNA by use of PCR with primers containing the desired restriction enzyme site.

In one embodiment, an expression construct comprising an α2M receptor sequence  
10 operably associated with regulatory regions can be directly introduced into appropriate host cells for expression and production of α2MR without further cloning (see, for example, U.S. Patent No. 5,580,859). The expression constructs may also contain DNA sequences that facilitate integration of the α2M receptor sequence into the genome of the host cell, e.g., via homologous recombination. In this instance, it is not necessary to employ an expression  
15 vector comprising a replication origin suitable for appropriate host cells in order to propagate and express the α2M receptor in the host cells.

Expression constructs containing cloned nucleotide sequence encoding the α2M receptor, an HSP, α2M, or other α2MR ligand, can be introduced into the host cell by a variety of techniques known in the art, including but not limited to, for prokaryotic cells,  
20 bacterial transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1:109-136), and for eukaryotic cells, calcium phosphate mediated transfection (Wigler et al., 1977, Cell 11:223-232), liposome-mediated transfection (Schaefer-Ridder et al., 1982, Science 215:166-168), electroporation (Wolff et al., 1987, Proc Natl Acad Sci 84:3344), and microinjection (Cappechi, 1980, Cell 22:479-488).

For long term, high yield production of properly processed α2M receptor, HSP, α2M, or other α2MR ligand, stable expression in mammalian cells is preferred. Cell lines that stably express the α2M receptor, HSP, α2M, or other α2MR ligand or α2MR-peptide complexes may be engineered by using a vector that contains a selectable marker. By way of example but not limitation, following the introduction of the expression constructs,  
30 engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the expression construct confers resistance to the selection and optimally allows cells to stably integrate the expression construct into their chromosomes and to grow in culture and to be expanded into cell lines. Such cells can be cultured for a long period of time while the desired gene product is  
35 expressed continuously.

The recombinant cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition. Alternatively, recombinant



antigenic cells may be cultured under conditions emulating the nutritional and physiological requirements of the cancer cell or infected cell. However, conditions for growth of recombinant cells may be different from those for expression of the  $\alpha 2M$  receptor, HSPs,  $\alpha 2M$ , or other  $\alpha 2MR$  ligand, or antigenic peptide.

5

### 5.1.2 PEPTIDE SYNTHESIS

An alternative to producing peptides and polypeptides comprising HSP,  $\alpha 2M$  receptor,  $\alpha 2M$  or other  $\alpha 2MR$  ligand sequences, by recombinant techniques is peptide synthesis. For example, a peptide corresponding to a portion of an HSP or an  $\alpha 2M$  peptide  
10 comprising the receptor-binding domain, which can be used as an antagonist in the therapeutic methods described herein, can be synthesized by use of a peptide synthesizer. Synthetic peptides corresponding to  $\alpha 2M$  receptor sequences useful for therapeutic methods described herein can also be produced synthetically. Conventional peptide synthesis may be used or other synthetic protocols well known in the art.

15 For example, peptides having the amino acid sequence of the  $\alpha 2M$  receptor, an HSP,  $\alpha 2M$ , or other  $\alpha 2MR$  ligand, or an analog, mutein, fragment, or derivative thereof, may be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, J. Am. Chem. Soc., 85:2149. During synthesis, N- $\alpha$ -protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its  
20 C-terminal and to an insoluble polymeric support i.e., polystyrene beads. The peptides are synthesized by linking an amino group of an N- $\alpha$ -deprotected amino acid to an  $\alpha$ -carboxyl group of an N- $\alpha$ -protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N- $\alpha$ -protecting groups  
25 include Boc which is acid labile and Fmoc which is base labile. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (*See*, Atherton, *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, and Bodanszky, 1993, Peptide Chemistry, A Practical Textbook, 2nd Ed., Springer-Verlag).

30 Purification of the resulting  $\alpha 2M$  receptor, HSP,  $\alpha 2M$ , or other  $\alpha 2MR$  ligand peptides is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

In addition, analogs and derivatives of  $\alpha 2M$  receptor, HSP,  $\alpha 2M$ , or other  $\alpha 2MR$   
35 ligand protein can be chemically synthesized. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the



α2M receptor, HSP, α2M, or other α2MR ligand sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general.

### 5.1.3 ANTIBODIES SPECIFIC FOR α2M RECEPTOR-HSP COMPLEXES

Described herein are methods for the production of antibodies capable of specifically recognizing α2M receptor epitopes, HSP-α2M receptor complex epitopes or epitopes of conserved variants or peptide fragments of the receptor or receptor complexes. Such antibodies are useful for therapeutic and diagnostic methods of the invention.

Such antibodies may include, but are not limited to, polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of an α2M receptor or HSP-α2M receptor complex in an biological sample. Such antibodies may also be utilized in conjunction with, for example, compound screening schemes, as described below, in Section 5.2, for the evaluation of the effect of test compounds on the interaction between HSPs and the α2M receptor.

Anti-α2M receptor complex antibodies may additionally be used as a method for the inhibition of abnormal receptor product activity. Thus, such antibodies may, be utilized as part of treatment methods for HSP-α2M receptor related disorders, *e.g.*, autoimmune disorders.

For the production of antibodies against α2M receptor or receptor complexes, various host animals may be immunized by injection with an α2M receptor or HSP-α2M receptor complex, or a portion thereof. An antigenic portion of α2M receptor or HSP-α2M receptor complex can be readily predicted by algorithms known in the art.

Host animals may include, but are not limited to rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and

potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as an  $\alpha 2\text{M}$  receptor or HSP- $\alpha 2\text{M}$  receptor complex, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with  $\alpha 2\text{M}$  receptor or HSP- $\alpha 2\text{M}$  receptor complex, or portion thereof, supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique of Kohler and Milstein, (1975, Nature 256, 495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor *et al.*, 1983, Immunology Today 4: 72; Cole *et al.*, 1983, Proc. Natl. Acad. Sci. USA 80, 2026-2030), and the EBV-hybridoma technique (Cole *et al.*, 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison, *et al.*, 1984, Proc. Natl. Acad. Sci., 81: 6851-6855; Neuberger, *et al.*, 1984, Nature 312: 604-608; Takeda, *et al.*, 1985, Nature, 314: 452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region (see, *e.g.*, Cabilly *et al.*, U.S. Patent No. 4,816,567; and Boss *et al.*, U.S. Patent No. 4,816,397, which are incorporated herein by reference in their entirety).

In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals (see PCT International Publication No. WO 89/12690, published December 12, 1989). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole *et al.*, 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). Techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger *et al.*, 1984, Nature 312:604-608; Takeda *et al.*,

1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for an  $\alpha$ 2M receptor-HSP complex together with genes from a human antibody molecule of appropriate biological activity can also be used; such antibodies are within the scope of this invention.

5 Humanized antibodies are also provided (see U.S. Patent No. 5,225,539 by Winter). An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, referred to as complementarity determining regions (CDRs). The extent of the framework region and CDRs have been precisely defined (see, "Sequences of Proteins of Immunological Interest", Kabat, E. *et al.*, U.S. Department of  
10 Health and Human Services (1983). Briefly, humanized antibodies are antibody molecules from non-human species having one or more CDRs from the non-human species and a framework region from a human immunoglobulin molecule. Such CDRS-grafted antibodies have been successfully constructed against various antigens, for example, antibodies against IL-2 receptor as described in Queen *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:10029; antibodies against the cell surface receptor CAMPATH as described in Riechmann *et al.*,  
15 1988, Nature 332:323; antibodies against hepatitis B in Co *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:2869; as well as against viral antigens of the respiratory syncytial virus in Tempest *et al.*, 1991, Bio-Technology 9:267. Humanized antibodies are most preferred for therapeutic use in humans.

20 Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242: 423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85: 5879-5883; and Ward *et al.*, 1989, Nature 334: 544-546) can be adapted to produce single chain antibodies against  $\alpha$ 2M receptor or HSP- $\alpha$ 2M receptor complexes, or portions thereof. Single chain antibodies are formed by linking the heavy and light chain  
25 fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')<sub>2</sub> fragments, which can be produced by pepsin digestion of the antibody molecule and the Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments.  
30 Alternatively, Fab expression libraries may be constructed (Huse *et al.*, 1989, Science, 246: 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to the  $\alpha$ 2M receptor can, in turn, be utilized to generate anti-idiotypic antibodies that "mimic" the  $\alpha$ 2M receptor, using techniques well known to those skilled in  
35 the art (see, *e.g.*, Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). For example antibodies which bind to the  $\alpha$ 2M receptor ECD and competitively inhibit the binding of HSPs to the  $\alpha$ 2M receptor can be used to generate

anti-idiotypes that "mimic" the ECD and, therefore, bind and neutralize HSPs. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize the native ligand and treat HSP- $\alpha$ 2M receptor-related disorders, such as immunological disorders, proliferative disorders, and infectious diseases.

5 Alternatively, antibodies to the  $\alpha$ 2M receptor that can act as agonists of the  $\alpha$ 2M receptor activity can be generated. Such antibodies will bind to the  $\alpha$ 2M receptor and activate the signal transducing activity of the receptor. In addition, antibodies that act as antagonist of the  $\alpha$ 2M receptor activity, *i.e.* inhibit the activation of the  $\alpha$ 2M receptor would be particularly useful for treating autoimmune disorders, proliferative disorders, such as  
10 cancer, and infectious diseases. Methods for assaying for such agonists and antagonists are described in detail in Section 5.2, below.

## 5.2 ASSAYS FOR THE IDENTIFICATION OF COMPOUNDS THAT INTERACT WITH THE $\alpha$ 2M RECEPTOR

15 The present invention is based on the discovery that the  $\alpha$ 2M receptor recognizes HSP-antigenic peptide complexes and transports them within the cell for the purpose of presenting such antigenic molecules to cells of the immune system and eliciting an immune response. Thus, methods for identifying compounds that interact with the receptor, or enhance or block the function of the receptor, are included in the invention. The present  
20 invention provides *in vitro* and *in vivo* assay systems, described in the subsections below, which can be used to identify compounds or compositions that interact with the  $\alpha$ 2M receptor, or modulate the activity of the  $\alpha$ 2M receptor and its interaction with HSPs or HSP-peptide complexes.

25 The invention provides screening methodologies useful in the identification of small molecules, proteins and other compounds which interact with the  $\alpha$ 2M receptor, or modulate the interaction of HSPs with the  $\alpha$ 2M receptor. Such compounds may bind the  $\alpha$ 2M receptor genes or gene products with differing affinities, and may serve as regulators of receptor activity *in vivo* with useful therapeutic applications in modulating the immune  
30 response. For example, certain compounds that inhibit receptor function may be used in patients to downregulate destructive immune responses which are caused by cellular release of HSPs.

Methods to screen potential agents for their ability to interact with the  $\alpha$ 2M receptor, or modulate  $\alpha$ 2M receptor expression and activity can be designed based on the inventor's  
35 discovery of the receptor and its role in HSP or HSP-peptide complex binding and recognition.  $\alpha$ 2M receptor protein, nucleic acids, and derivatives can be used in screening assays to detect molecules that specifically bind to HSP proteins, derivatives, or nucleic

acids, and thus have potential use as agonists or antagonists of the  $\alpha$ 2M receptor, to modulate the immune response. In a preferred embodiment, such assays are performed to screen for molecules with potential utility as anti-autoimmune disease, anti-cancer and anti-infective drugs (such as anti-viral drugs and antibiotic drugs), or lead compounds for drug  
5 development. For example, recombinant cells expressing  $\alpha$ 2M receptor nucleic acids can be used to recombinantly produce  $\alpha$ 2M receptor in these assays, to screen for molecules that interfere with the binding of HSPs to the  $\alpha$ 2M receptor. Similar methods can be used to screen for molecules that bind to the  $\alpha$ 2M receptor derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

10 Compounds capable of specifically binding the  $\alpha$ 2M receptor can be useful for immunotherapy. In one embodiment, an assay is disclosed for identifying compounds that specifically bind the  $\alpha$ 2M receptor comprising: (a) contacting an  $\alpha$ 2M receptor with one or more test compounds under conditions conducive to binding; and (b) identifying one or more test compounds which specifically bind to the  $\alpha$ 2M receptor, such that a compound capable  
15 of specifically binding the  $\alpha$ 2M receptor is identified as a compound useful for immunotherapy.

Another method encompassed by the invention for identifying a compound useful for immunotherapy involves identifying a compound which modulates the binding of an  $\alpha$ 2M receptor ligand to the  $\alpha$ 2M receptor. The term " $\alpha$ 2M receptor ligand" as used herein, refers  
20 to an molecule capable of binding to the  $\alpha$ 2M receptor. Such  $\alpha$ 2M receptor ligands include, but are not limited to,  $\alpha$ 2M and  $\alpha$ 2M complexes, heat shock proteins and heat shock protein complexes, lipoprotein complexes, lactoferrin, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), and exotoxins. Such ligands are typically endocytosed by cell upon binding to the  $\alpha$ 2M receptor. The method comprises the steps of:  
25 (a) contacting an  $\alpha$ 2M receptor with an  $\alpha$ 2M receptor ligand, or fragment, or analog, derivative or mimetic thereof, in the presence of one or more test compound; and (b) measuring the amount of  $\alpha$ 2M receptor ligand, or fragment, analog, derivative or mimetic thereof, bound to the  $\alpha$ 2M receptor, such that if the amount of bound  $\alpha$ 2M receptor ligand measured in (b) differs from the amount of bound  $\alpha$ 2M receptor measured in the absence of  
30 the test compound, then a compound useful for immunotherapy that modulates the binding of an  $\alpha$ 2M receptor ligand to the  $\alpha$ 2M receptor is identified.

In another embodiment, a method for identifying a compound useful for immunotherapy which modulates the interaction between the  $\alpha$ 2M receptor and an  $\alpha$ 2M receptor ligand is provided by the invention. This method comprises the steps of: (a)  
35 contacting an  $\alpha$ 2M receptor with one or more test compounds; and (b) measuring the level of  $\alpha$ 2M receptor activity or expression, such that if the level of activity or expression measured in (b) differs from the level of  $\alpha$ 2M receptor activity in the absence of one or more test



compounds, then a compound that modulates the interaction between the  $\alpha$ 2M receptor and an  $\alpha$ 2M receptor ligand is identified.

In another embodiment, an assay for identifying a compound that modulates an HSP- $\alpha$ 2M receptor-mediated process is disclosed. This assay comprises: (a) contacting a test compound with an HSP and an  $\alpha$ 2M receptor; and (b) measuring the level of  $\alpha$ 2M receptor activity or expression, such that if the level of activity or expression measured in (b) differs from the level of  $\alpha$ 2M receptor activity in the absence of the test compound, then a compound that modulates an HSP- $\alpha$ 2M receptor-mediated process is identified. In another embodiment, in which the compound identified is an antagonist which interferes with the interaction of the HSP with the  $\alpha$ 2M receptor, the method further comprises the step of determining whether the level interferes with the interaction of the HSP and the  $\alpha$ 2M receptor.

In another embodiment, a cell-based method for identifying a compound that modulates an HSP- $\alpha$ 2M receptor-mediated process is described. This method comprises the following steps: (a) contacting a test compound with a heat shock protein and an  $\alpha$ 2M receptor-expressing cell; and (b) measuring the level of  $\alpha$ 2M receptor activity or expression in the cell, such that if the level of activity or expression measured in (b) differs from the level of  $\alpha$ 2M receptor activity in the absence of the test compound, then a compound that modulates an HSP- $\alpha$ 2M receptor-mediated process is identified.

In another embodiment, a receptor-ligand binding assay for identifying a compound that interacts with  $\alpha$ 2MR, or modulates the binding of an HSP to  $\alpha$ 2MR. One such method comprises: (a) contacting an HSP with an  $\alpha$ 2M receptor, or fragment, or analog, derivative or mimetic thereof, in the presence of a test compound; and (b) measuring the amount of heat shock protein bound to the  $\alpha$ 2M receptor, or fragment, analog, derivative or mimetic thereof, such that if the amount of bound heat shock protein measured in (b) differs from the amount of bound heat shock protein measured in the absence of the test compound, then a compound that modulates the binding of an HSP to the  $\alpha$ 2M receptor is identified.

In another embodiment, a method for identifying a compound that modulates antigen presentation by  $\alpha$ 2MR-expressing cells is provided by the invention. In one embodiment, such a method comprises: (a) adding one or more test compounds to a mixture of  $\alpha$ 2MR-expressing cells and a complex comprising an  $\alpha$ 2MR ligand and an antigenic molecule, under conditions conducive to  $\alpha$ 2MR-mediated endocytosis; (2) measuring the level of stimulation of antigen-specific cytotoxic T cells by the  $\alpha$ 2MR-expressing cells, such that if the level measured in (b) differs from the level of said stimulation in the absence of the one or more test compounds, then a compound that modulates antigen presentation by  $\alpha$ 2MR-expressing cells is identified. In another embodiment, a test compound is added to a mixture of  $\alpha$ 2MR-expressing cells and a complex consisting essentially of an HSP noncovalently

associated with an antigenic molecule, under conditions conducive to  $\alpha$ 2MR-mediated endocytosis; and the level of stimulation of antigen-specific cytotoxic T cells by the  $\alpha$ 2MR-expressing cells is measured, such that if the level measured differs from the level of said stimulation in the absence of the test compound, then a compound that modulates HSP-mediated antigen presentation by  $\alpha$ 2MR-expressing cells is identified.

The assays of the present invention may be first optimized on a small scale (*i.e.*, in test tubes), and then scaled up for high-throughput assays. In various embodiments, the *in vitro* screening assays of the present invention may be performed using purified components or cell lysates. In other embodiments, the screening assays may be carried out in intact cells in culture and in animal models. In accordance with the present invention, test compounds which are shown to modulate the activity of the  $\alpha$ 2M receptor as described herein *in vitro*, will further be assayed *in vivo*, including cultured cells and animal models to determine if the test compound has the similar effects *in vivo* and to determine the effects of the test compound on antigen presentation, cytokine release, intracellular  $\text{Ca}^{++}$  release, T-cell cytotoxicity, tumor progression, the accumulation or degradation of positive and negative regulators, cellular proliferation, *etc.*

#### 5.2.1 $\alpha$ 2M RECEPTOR-LIGAND BINDING ASSAYS

The screening assays, described herein, can be used to identify compounds and compositions, including peptides and organic, non-protein molecules that interact with the  $\alpha$ 2M receptor, or that modulate the interaction between HSPs and the  $\alpha$ 2M receptor. Recombinant, synthetic, and otherwise exogenous compounds may have binding capacity and, therefore, may be candidates for pharmaceutical agents. Alternatively, the proteins and compounds include endogenous cellular components which interact with the identified genes and proteins *in vivo*. Such endogenous components may provide new targets for pharmaceutical and therapeutic interventions.

Thus, in a preferred embodiment, both naturally occurring and/or synthetic compounds (*e.g.*, libraries of small molecules or peptides), may be screened for interacting with  $\alpha$ 2M receptor and/or modulating  $\alpha$ 2M receptor activity. In another series of embodiments, cell lysates or tissue homogenates may be screened for proteins or other compounds which bind to one of the normal or mutant  $\alpha$ 2M receptor genes and  $\alpha$ 2M receptor polypeptides.

The screening assays described herein may be used to identify small molecules, peptides or proteins, or derivatives, analogs and fragments thereof, that interact with and/or modulate the interaction of HSPs with the  $\alpha$ 2M receptor. Such compounds may be used as agonists or antagonists of the uptake of  $\alpha$ 2M receptor ligands, such as HSPs and HSP

complexes, by the cell surface receptor. For example, compounds that modulate the  $\alpha$ 2M receptor-ligand interaction include, but are not limited to, compounds that bind to the  $\alpha$ 2M receptor, thereby either inhibiting (antagonists) or enhancing (agonists) the binding of ligands, such as HSPs and HSP complexes, to the receptor, as well as compounds that bind to the ligand, such as for example, HSPs, thereby preventing or enhancing binding of ligand to the receptor. Compounds that affect  $\alpha$ 2M receptor gene activity (by affecting  $\alpha$ 2M receptor gene expression, including molecules, *e.g.*, proteins or small organic molecules, that affect transcription or interfere with splicing events so that expression of the full length or truncated forms of  $\alpha$ 2M receptor can be modulated) can also be identified in the screens of the invention. Further, it should be noted that the assays described can also identify compounds that modulate  $\alpha$ 2M receptor ligand, for example HSP, uptake by  $\alpha$ 2M receptor (*e.g.*, compounds which affect downstream signaling in the  $\alpha$ 2M receptor signal transduction pathway). The identification and use of such compounds which affect signaling events downstream of the  $\alpha$ 2M receptor and thus modulate effects of the receptor on the immune response are within the scope of the invention.

Compounds that affect the  $\alpha$ 2M receptor gene activity (by affecting the  $\alpha$ 2M receptor gene expression, including molecules, *e.g.*, proteins or small organic molecules, that affect transcription or interfere with splicing events so that expression of the full length or the truncated form of the  $\alpha$ 2M receptor can be modulated) can also be identified in the screens of the invention. However, it should be noted that the assays described can also identify compounds that modulate the  $\alpha$ 2M receptor signal transduction (*e.g.*, compounds which affect downstream signaling events, such as inhibitors or enhancers of endocytic activity which is activated by ligand binding to the  $\alpha$ 2M receptor). The identification and use of such compounds which affect signaling events downstream of the  $\alpha$ 2M receptor and thus modulate effects of the  $\alpha$ 2M receptor on the allergenic response are within the scope of the invention.

The screening assays described herein are designed to detect compounds that modulate, *i.e.* interfere with or enhance, ligand-receptor interactions, including HSP- $\alpha$ 2M receptor interactions. As described in detail below, such assays are functional assays, such as binding assays, that can be adapted to a high-throughput screening methodologies.

Binding assays can be used to identify compounds that modulate the interaction between ligands, for example, HSPs, and the  $\alpha$ 2M receptor. In one aspect of the invention the screens may be designed to identify compounds that disrupt the interaction between the  $\alpha$ 2M receptor and a ligand, such as, for example, HSPs or peptides derived from an HSP,  $\alpha$ 2M, or another  $\alpha$ 2M receptor ligand. Such compounds will be useful as lead compounds for antagonists of HSP- $\alpha$ 2M receptor-related disorders and conditions, such as immune disorders, proliferative disorders, and infectious diseases.

Binding assays may be performed either as direct binding assays or as competition binding assays. In a direct binding assay, a test compound is tested for binding either to the  $\alpha$ 2M receptor or to an  $\alpha$ 2M receptor ligand, such as an HSP. Then, in a second step, the test compound is tested for its ability to modulate the ligand- $\alpha$ 2M receptor interaction.

- 5 Competition binding assays, on the other hand, assess the ability of a test compound to compete with a ligand, *i.e.* an HSP, for binding to the  $\alpha$ 2M receptor.

In a direct binding assay, either the ligand and/or the  $\alpha$ 2M receptor is contacted with a test compound under conditions that allow binding of the test compound to the ligand or the receptor. The binding may take place in solution or on a solid surface. Preferably, the  
10 test compound is previously labeled for detection. Any detectable compound may be used for labeling, such as but not limited to, a luminescent, fluorescent, or radioactive isotope or group containing same, or a nonisotopic label, such as an enzyme or dye. After a period of incubation sufficient for binding to take place, the reaction is exposed to conditions and manipulations that remove excess or non-specifically bound test compound. Typically, it  
15 involves washing with an appropriate buffer. Finally, the presence of a ligand-test compound (*e.g.*, HSP-test compound) or a the  $\alpha$ 2M receptor-test compound complex is detected.

In a competition binding assay, test compounds are assayed for their ability to disrupt or enhance the binding of the ligand (*e.g.*, HSP) to the  $\alpha$ 2M receptor. Labeled ligand (*e.g.*,  
20 HSP) may be mixed with the  $\alpha$ 2M receptor or fragment or derivative thereof, and placed under conditions in which the interaction between them would normally occur, with and without the addition of the test compound. The amount of labeled ligand (*e.g.*, HSP) that binds the  $\alpha$ 2M receptor may be compared to the amount bound in the presence or absence of test compound.

25 In a preferred embodiment, to facilitate complex formation and detection, the binding assay is carried out with one or more components immobilized on a solid surface. In various embodiments, the solid support could be, but is not restricted to, polycarbonate, polystyrene, polypropylene, polyethylene, glass, nitrocellulose, dextran, nylon, polyacrylamide and agarose. The support configuration can include beads, membranes,  
30 microparticles, the interior surface of a reaction vessel such as a microtiter plate, test tube or other reaction vessel. The immobilization of the  $\alpha$ 2M receptor, or other component, can be achieved through covalent or non-covalent attachments. In one embodiment, the attachment may be indirect, *i.e.* through an attached antibody. In another embodiment, the  $\alpha$ 2M receptor and negative controls are tagged with an epitope, such as glutathione S-transferase (GST) so  
35 that the attachment to the solid surface can be mediated by a commercially available antibody such as anti-GST (Santa Cruz Biotechnology).

For example, such an affinity binding assay may be performed using a the  $\alpha 2M$  receptor which is immobilized to a solid support. Typically, the non-mobilized component of the binding reaction, in this case either ligand (e.g., HSP) or the test compound, is labeled to enable detection. A variety of labeling methods are available and may be used, such as  
5 luminescent, chromophore, fluorescent, or radioactive isotope or group containing same, and nonisotopic labels, such as enzymes or dyes. In a preferred embodiment, the test compound is labeled with a fluorophore such as fluorescein isothiocyanate (FITC, available from Sigma Chemicals, St. Louis).

The labeled test compounds, or ligand (e.g., HSP) plus test compounds, are then  
10 allowed to contact with the solid support, under conditions that allow specific binding to occur. After the binding reaction has taken place, unbound and non-specifically bound test compounds are separated by means of washing the surface. Attachment of the binding partner to the solid phase can be accomplished in various ways known to those skilled in the art, including but not limited to chemical cross-linking, non-specific adhesion to a plastic  
15 surface, interaction with an antibody attached to the solid phase, interaction between a ligand attached to the binding partner (such as biotin) and a ligand-binding protein (such as avidin or streptavidin) attached to the solid phase, and so on.

Finally, the label remaining on the solid surface may be detected by any detection method known in the art. For example, if the test compound is labeled with a fluorophore, a  
20 fluorimeter may be used to detect complexes.

Preferably, the  $\alpha 2M$  receptor is added to binding assays in the form of intact cells that express the  $\alpha 2M$  receptor, or isolated membranes containing the  $\alpha 2M$  receptor. Thus, direct binding to the  $\alpha 2M$  receptor or the ability of a test compound to modulate a ligand- $\alpha 2M$  receptor complex (e.g., HSP-  $\alpha 2M$  receptor complex) may be assayed in intact cells in  
25 culture or in animal models in the presence and absence of the test compound. A labeled ligand (e.g., HSP) may be mixed with cells that express the  $\alpha 2M$  receptor, or to crude extracts obtained from such cells, and the test compound may be added. Isolated membranes may be used to identify compounds that interact with the  $\alpha 2M$  receptor. For example, in a typical experiment using isolated membranes, cells may be genetically engineered to express  
30 the  $\alpha 2M$  receptor. Membranes can be harvested by standard techniques and used in an *in vitro* binding assay. Labeled ligand (e.g.,  $^{125}I$ -labeled HSP) is bound to the membranes and assayed for specific activity; specific binding is determined by comparison with binding assays performed in the presence of excess unlabeled (cold) ligand. Alternatively, soluble  $\alpha 2M$  receptor may be recombinantly expressed and utilized in non-cell based assays to  
35 identify compounds that bind to the  $\alpha 2M$  receptor. The recombinantly expressed  $\alpha 2M$  receptor polypeptides or fusion proteins containing the extracellular domain (ECD) of the  $\alpha 2M$  receptor, or one or more subdomains thereof, can be used in the non-cell based



screening assays. Alternatively, peptides corresponding to one or more of the CDs of the  $\alpha$ 2M receptor, or fusion proteins containing one or more of the CDs of the  $\alpha$ 2M receptor can be used in non-cell based assay systems to identify compounds that bind to the cytoplasmic portion of the  $\alpha$ 2M receptor; such compounds may be useful to modulate the signal transduction pathway of the  $\alpha$ 2M receptor. In non-cell based assays the recombinantly expressed the  $\alpha$ 2M receptor is attached to a solid substrate such as a test tube, microtiter well or a column, by means well known to those in the art (see Ausubel *et al.*, *supra*). The test compounds are then assayed for their ability to bind to the  $\alpha$ 2M receptor.

Alternatively, the binding reaction may be carried out in solution. In this assay, the labeled component is allowed to interact with its binding partner(s) in solution. If the size differences between the labeled component and its binding partner(s) permit such a separation, the separation can be achieved by passing the products of the binding reaction through an ultrafilter whose pores allow passage of unbound labeled component but not of its binding partner(s) or of labeled component bound to its partner(s). Separation can also be achieved using any reagent capable of capturing a binding partner of the labeled component from solution, such as an antibody against the binding partner, a ligand-binding protein which can interact with a ligand previously attached to the binding partner, and so on.

In a one embodiment, for example, a phage library can be screened by passing phage from a continuous phage display library through a column containing purified  $\alpha$ 2M receptor, or derivative, analog, fragment, or domain, thereof, linked to a solid phase, such as plastic beads. By altering the stringency of the washing buffer, it is possible to enrich for phage that express peptides with high affinity for the  $\alpha$ 2M receptor. Phage isolated from the column can be cloned and the affinities of the short peptides can be measured directly. Sequences for more than one oligonucleotide can be combined to test for even higher affinity binding to the  $\alpha$ 2M receptor. Knowing which amino acid sequences confer the strongest binding to the  $\alpha$ 2M receptor, computer models can be used to identify the molecular contacts between the  $\alpha$ 2M receptor and the test compound. This will allow the design of non-protein compounds which mimic those contacts. Such a compound may have the same activity of the peptide and can be used therapeutically, having the advantage of being efficient and less costly to produce.

In another specific embodiment of this aspect of the invention, the solid support is membranes containing the  $\alpha$ 2M receptor attached to a microtiter dish. Test compounds, for example, cells that express library members are cultivated under conditions that allow expression of the library members in the microtiter dish. Library members that bind to the protein (or nucleic acid or derivative) are harvested. Such methods, are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes *et al.*, 1992,

BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment of the present invention, interactions between the  $\alpha$ 2M receptor or ligand (e.g., HSP) and a test compound may be assayed *in vitro*. Known or unknown molecules are assayed for specific binding to the  $\alpha$ 2M receptor nucleic acids, proteins, or derivatives under conditions conducive to binding, and then molecules that specifically bind to the  $\alpha$ 2M receptor are identified. The two components can be measured in a variety of ways. One approach is to label one of the components with an easily detectable label, place it together with a test component(s) under conditions that allow binding to occur, perform a separation step which separates bound labeled component from unbound labeled component, and then measure the amount of bound component. In one embodiment, the  $\alpha$ 2M receptor can be labeled and added to a test agent, using conditions that allow binding to occur. Binding of the test agent can be determined using polyacrylamide gel analysis to compare complexes formed in the presence and absence of the test agent.

In yet another embodiment, binding of ligand (e.g., HSP) to the  $\alpha$ 2M receptor may be assayed in intact cells in animal models. A labeled ligand (e.g., HSP) may be administered directly to an animal, with and without a test compound. Uptake of the ligand (e.g., HSP) may be measured in the presence and the absence of test compound. For these assays, host cells to which the test compound is added may be genetically engineered to express the  $\alpha$ 2M receptor and/or ligand (e.g., HSP), which may be transient, induced or constitutive, or stable. For the purposes of the screening methods of the present invention, a wide variety of host cells may be used including, but not limited to, tissue culture cells, mammalian cells, yeast cells, and bacteria. Mammalian cells such as macrophages or other cells that express the  $\alpha$ 2M receptor, *i.e.*, cells of the monocytic lineage, liver parenchymal cells, fibroblasts, keratinocytes, neuronal cells, and placental syncytiotrophoblasts, may be a preferred cell type in which to carry out the assays of the present invention. Bacteria and yeast are relatively easy to cultivate but process proteins differently than mammalian cells.

#### 5.2.2 $\alpha$ 2M RECEPTOR ACTIVITY ASSAYS

After identification of a test compound that interacts with, or modulates the interaction of a ligand (e.g., HSP) with  $\alpha$ 2MR, the test compound can be further characterized to measure its effect on  $\alpha$ 2MR activity and the ligand- $\alpha$ 2MR endocytic signaling pathway. For example, the test compound may be characterized by testing its effect on ligand (e.g., HSP) / $\alpha$ 2MR cellular activity *in vivo*. Such assays include downstream signaling assays, antigen presentation assays, assays for antigen-specific activation of cytotoxic T cells, and the like.

In various embodiments, a candidate compound identified in a primary assay may be tested for its effect on innate  $\alpha$ 2MR signaling activity. For example, downstream signaling effects of  $\alpha$ 2M receptor activation which can be assayed include, but are not limited to: enhanced locomotion and chemotaxis of macrophages (Forrester *et al.*, 1983, Immunology 50: 251-259), down regulation of proteinase synthesis, and elevation of intracellular calcium, inositol phosphates and cyclic AMP (Misra *et al.*, 1993, Biochem. J., 290:885-891). Other innate immune responses that can be tested are release of cytokines (*i.e.*, IL-12, IL-1 $\beta$ , GM-CSF, and TNF $\alpha$ ). Thus, as secondary assays, any identified candidate compound can be tested for changes in such activities in the presence and absence.

For example, in one embodiment, a chemotaxis assay can be used to further characterize a candidate identified by a primary screening assay. It is known that  $\alpha$ 2M modified by protease interaction can induce directional migration of cells towards their ligand. A number of techniques can be used to test chemotactic migration *in vitro* (see, *e.g.*, Leonard *et al.*, 1995, "Measurement of  $\alpha$  and  $\beta$  Chemokines", in Current Protocols in Immunology, 6.12.1-6.12.28, Ed. Coligan *et al.*, John Wiley & Sons, Inc. 1995). For example, in one embodiment, a candidate compound can be tested for its ability to modulate the ability of  $\alpha$ 2MR to induce migration of cells that express the receptor using a chemokine gradient in a multiwell Boyden chemotaxis chamber. In a specific example of this method, a serial dilution of a ligand (*e.g.*, an HSP) /  $\alpha$ 2MR antagonist or agonist test compound identified in the primary screen is placed in the bottom wells of the Boyden chemotaxis chamber. A constant amount of ligand is also added to the dilution series. As a control, at least one aliquot contains only ligand (*e.g.*, HSP). The contribution of the antagonist or agonist compound to the chemotactic activity of  $\alpha$ 2MR is measured by comparing number of migrating cells on the lower surface of the membrane filter of the aliquots containing only ligand (*e.g.*, HSP), with the number of cells in aliquots containing test compound and ligand (*e.g.*, HSP). If addition of the test compound to the ligand (*e.g.*, HSP) solution results in a decrease in the number of cells detected the membrane relative to the number of cells detected using a solution containing only ligand (*e.g.*, HSP), then an antagonist of ligand (*e.g.*, HSP) induction of chemotactic activity of  $\alpha$ 2MR-expressing cells is identified.

Elevation in intracellular ionized calcium concentration ( $[Ca^{2+}]_i$ ) is also an indicator of  $\alpha$ 2MR activation (Misra *et al.*, 1993, *supra*). Thus, in another embodiment, calcium flux assays can be used as secondary screens to further characterize modulators of ligand- $\alpha$ 2MR interactions. Intracellular calcium ion concentration can be measured in cells that express the  $\alpha$ 2M receptor in the presence of the ligand, in the presence and the absence of a test compound. For example, calcium mobilization can be detected and measured by flow cytometry, by labeling with fluorescent dyes that are trapped intracellularly. A fluorescent dye such as Indo-1 exhibits a change in emission spectrum upon binding calcium, the ratio of

fluorescence produced by the calcium-bound dye to that produce by the unbound dye may be used to estimate the intracellular calcium concentration. In a specific embodiment, cells are incubated in a cuvette in media containing Indo-1 at 37°C and are excited, and fluorescence is measured using a fluorimeter (Photon Technology Corporation, International). The ligand is added at a specific time point, in the presence and the absence of a test compound, EGTA is added to the cuvette to release and chelate total calcium, and the response is measured. Binding of ligand results in increased intracellular  $\text{Ca}^{2+}$  concentration in cells that express  $\alpha 2\text{MR}$ . An agonist results in a relative increased intracellular  $\text{Ca}^{2+}$  concentration, whereas an antagonist results in a relative decreased intracellular  $\text{Ca}^{2+}$  concentration

In other embodiments, antigen-specific response assays may be used to detect the effect of a candidate compound on presentation of antigenic molecule by an  $\alpha 2\text{MR}$  ligand, for example an HSP or HSP complex. For example, an antigen presentation assay may be performed to determine the effect of a compound *in vivo* on the uptake of complexes capable of interacting with the  $\alpha 2\text{M}$  receptor, *e.g.*, HSP-antigenic molecule complexes, by cells expressing the  $\alpha 2\text{M}$  receptor. Such re-presentation assays are known in the art, and have been described previously (Suto and Srivastava, 1995, Science 269:1585-1588). For example, in one embodiment, antigen presenting cells, such as a macrophage cell line (*e.g.*, RAW264.7), are mixed with antigen-specific T cells in media, using approximately 10,000 cells of each type at approximately a 1:1 ratio. Complexes of HSP (10  $\mu\text{g}/\text{ml}$ ) and a peptide antigen, as well as test compound, is added to the cells and the culture is incubated for approximately 20 hours. Stimulation of T cells may then be measured in the presence and absence of test compound.

In another embodiment, antigen-specific T cell stimulation may be assayed. In one embodiment an IFN- $\gamma$  release assay may be used. After washing, cells are fixed, permeabilized, and reacted with dye-labeled antibodies reactive with human IFN- $\gamma$  (PE- anti-IFN- $\gamma$ ). Samples are analyzed by flow cytometry using standard techniques. Alternatively, a filter immunoassay, ELISA (enzyme linked immunosorbent assay), or enzyme-linked immunospot assay (ELISPOT) assay, may be used to detect specific cytokines produced by an activated T cell. In one embodiment, for example, a nitrocellulose-backed microtiter plate is coated with a purified cytokine-specific primary antibody, *i.e.*, anti-IFN- $\gamma$ , and the plate is blocked to avoid background due to nonspecific binding of other proteins. A sample of APC cells stimulated with antigen is diluted onto the wells of the microtiter plate. A labeled, *e.g.*, biotin-labeled, secondary anti-cytokine antibody is added. The antibody cytokine complex can then be detected, *i.e.*, by enzyme-conjugated streptavidin – cytokine-secreting cells will appear as “spots” by visual, microscopic, or electronic detection methods. In another embodiment, “tetramer staining” assay (Altman *et al.*, 1996, Science 274: 94-96) may be used to identify antigen-specific T-cells. For example, an MHC molecule containing a

specific peptide antigen, such as a tumor-specific antigen, is multimerized to make soluble peptide tetramers and labeled, for example, by complexing to streptavidin. The MHC-peptide antigen complex is then mixed with a population of stimulated T cells. Biotin is then used to stain T cells which recognize and bind to the MHC-antigen complex.

### 5.2.3 COMPOUNDS THAT CAN BE SCREENED IN ACCORDANCE WITH THE INVENTION

The screening assays described herein may be used to identify small molecules, peptides or proteins, or derivatives, analogs and fragments thereof, that interact with, or modulate the interaction of a ligand (*e.g.*, HSP) with the  $\alpha$ 2M receptor. The compounds which may be screened in accordance with the invention include, but are not limited to small molecules, peptides, antibodies and fragments thereof, and other organic compounds (*e.g.*, peptidomimetics) that bind to the ECD of the  $\alpha$ 2M receptor and either inhibit the activity triggered by the natural ligand (*i.e.*, antagonists) or mimic the activity triggered by the natural ligand (*i.e.*, agonists), as well as small molecules, peptides, antibodies or fragments thereof, and other organic compounds. In one embodiment, such compounds include sequences of the  $\alpha$ 2M receptor, such as the ECD of the  $\alpha$ 2M receptor (or a portion thereof), which can bind to and "neutralize" natural ligands, such as HSPs,  $\alpha$ 2M, LDL, *etc.* In another embodiment, such compounds include ligand sequences, such as HSP sequences and/or  $\alpha$ 2M sequences, which can bind to the active site of the  $\alpha$ 2M receptor, and block its activity.

Compounds that may be used for screening include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, *e.g.*, Lam *et al.*, 1991, Nature 354:82-84; Houghten *et al.*, 1991, Nature 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; see, *e.g.*, Songyang *et al.*, 1993, Cell 72:767-778), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, F(ab')<sub>2</sub> and FAb expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

In one embodiment of the present invention, peptide libraries may be used as a source of test compounds that can be used to screen for modulators of  $\alpha$ 2MR interactions, such as HSP-  $\alpha$ 2M receptor. Diversity libraries, such as random or combinatorial peptide or nonpeptide libraries can be screened for molecules that specifically bind to the  $\alpha$ 2M receptor. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.



Examples of chemically synthesized libraries are described in Fodor *et al.*, 1991, Science 251:767-773; Houghten *et al.*, 1991, Nature 354:84-86; Lam *et al.*, 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop *et al.*, 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten *et al.*, 1992, Biotechniques 13:412; Jayawickreme *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott & Smith, 1990, Science 249:386-390; Devlin *et al.*, 1990, Science, 249:404-406; Christian *et al.*, 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay *et al.*, 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

By way of examples of nonpeptide libraries, a benzodiazepine library (*see e.g.*, Bunin *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh *et al.* (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley & Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott & Smith, 1990, Science 249:386-390; Fowlkes *et al.*, 1992; BioTechniques 13:422-427; Oldenburg *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu *et al.*, 1994, Cell 76:933-945; Staudt *et al.*, 1988, Science 241:577-580; Bock *et al.*, 1992, Nature 355:564-566; Tuerk *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington *et al.*, 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner *et al.*; Rebar & Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In another embodiment of the present invention, the screening may be performed by adding the labeled ligand (*e.g.*, HSP) to *in vitro* translation systems such as a rabbit reticulocyte lysate (RRL) system and then proceeding with *in vitro* priming reaction. *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

Compounds that can be tested and identified methods described herein can include, but are not limited to, compounds obtained from any commercial source, including Aldrich

(Milwaukee, WI 53233), Sigma Chemical (St. Louis, MO), Fluka Chemie AG (Buchs, Switzerland) Fluka Chemical Corp. (Ronkonkoma, NY;), Eastman Chemical Company, Fine Chemicals (Kingsport, TN), Boehringer Mannheim GmbH (Mannheim, Germany), Takasago (Rockleigh, NJ), SST Corporation (Clifton, NJ), Ferro (Zachary, LA 70791), Riedel-deHaen  
5 Aktiengesellschaft (Seelze, Germany), PPG Industries Inc., Fine Chemicals (Pittsburgh, PA 15272). Further any kind of natural products may be screened using the methods of the invention, including microbial, fungal, plant or animal extracts.

Furthermore, diversity libraries of test compounds, including small molecule test compounds, may be utilized. For example, libraries may be commercially obtained from  
10 Specs and BioSpecs B.V. (Rijswijk, The Netherlands), Chembridge Corporation (San Diego, CA), Contract Service Company (Dolgoprudny, Moscow Region, Russia), Comgenex USA Inc. (Princeton, NJ), Maybridge Chemicals Ltd. (Cornwall PL34 OHW, United Kingdom), and Asinex (Moscow, Russia).

Still further, combinatorial library methods known in the art, can be utilize, including,  
15 but not limited to: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of  
20 compounds (Lam, 1997, Anticancer Drug Des. 12:145). Combinatorial libraries of test compounds, including small molecule test compounds, can be utilized, and may, for example, be generated as disclosed in Eichler & Houghten, 1995, Mol. Med. Today 1:174-180; Dolle, 1997, Mol. Divers. 2:223-236; and Lam, 1997, Anticancer Drug Des. 12:145-167.

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:6909; Erb *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann *et al.*, 1994, J. Med. Chem. 37:2678; Cho  
25 *et al.*, 1993, Science 261:1303; Carrell *et al.*, 1994, Angew. Chem. Int. Ed. Engl. 33:2059; Carell *et al.*, 1994, Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop *et al.*, 1994, J. Med.  
30 Chem. 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten, 1992, BioTechniques 13:412-421), or on beads (Lam, 1991, Nature 354:82-84), chips (Fodor, 1993, Nature 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull *et al.*, 1992, Proc. Natl. Acad. Sci.  
35 USA 89:1865-1869) or phage (Scott and Smith, 1990, Science 249:386-390; Devlin, 1990, Science 249:404-406; Cwirla *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:6378-6382; and Felici, 1991, J. Mol. Biol. 222:301-310).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley & Smith, 1989, *Adv. Exp. Med. Biol.* 251:215-218; Scott & Smith, 1990, *Science* 249:386-390; Fowlkes *et al.*, 1992, *BioTechniques* 13:422-427; Oldenburg *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:5393-5397; Yu *et al.*, 1994, *Cell* 76:933-945; Staudt *et al.*, 1988, *Science* 241:577-580; Bock *et al.*, 1992, *Nature* 355:564-566; Tuerk *et al.*, 1992, *Proc. Natl. Acad. Sci. USA* 89:6988-6992; Ellington *et al.*, 1992, *Nature* 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner *et al.*; Rebar & Pabo, 1993, *Science* 263:671-673; and PCT Publication No. WO 94/18318.

### 5.3 IDENTIFICATION OF FRAGMENTS OF THE $\alpha$ 2M RECEPTOR AND/OR $\alpha$ 2M RECEPTOR LIGANDS, SUCH AS HSPTS, USEFUL FOR IMMUNOTHERAPY

The invention also encompasses methods for identifying ligand-binding  $\alpha$ 2MR fragments (such as "HSP-binding domains"), and analogs, muteins, or derivatives thereof, which are capable of binding to, and uptake of,  $\alpha$ 2MR ligand-antigenic peptide, such as HSP-antigenic peptide complexes. Such ligand-binding  $\alpha$ 2MR fragment, *e.g.*, HSP-binding domains, can then be tested for activity *in vivo* and *in vitro* using the  $\alpha$ 2M receptor/ligand binding assays, described in Section 5.2.1, above. In one embodiment, such a method for identifying an  $\alpha$ 2MR fragment capable of binding a heat shock protein comprises the steps of: (a) contacting a heat shock protein with one or more  $\alpha$ 2MR fragments; and (b) identifying an  $\alpha$ 2MR polypeptide fragment which specifically binds to the heat shock protein.

Ligand-binding domains, *e.g.*, HSP-binding domains, of the  $\alpha$ 2MR capable of binding ligand-antigenic peptide complexes, such as HSP-antigenic peptide complexes, and can be further tested for activity using either *in vivo* binding assays, re-presentation assays, or CTL assays, such as those described in Section 5.2.2, above. For example, one such method for identifying an  $\alpha$ 2MR fragment capable of inducing an HSP- $\alpha$ 2M receptor-mediated process comprises the steps of: (a) contacting a heat shock protein with cell expressing  $\alpha$ 2MR fragment; and (b) measuring the level of  $\alpha$ 2MR activity in the cell, such that if the level of the HSP- $\alpha$ 2M receptor-mediated process or activity measured in (b) is greater than the level of  $\alpha$ 2MR activity in the absence of the  $\alpha$ 2MR fragment, then an  $\alpha$ 2MR fragment capable of inducing an HSP- $\alpha$ 2M receptor-mediated process is identified. Depending on their behavior in such assays, such molecules can be used to either enhance or, alternatively, block the function of the receptor when administered or expressed *in vivo*. For example, these assays can be used to identify  $\alpha$ 2MR HSP-binding domains which can bind HSP-

antigen complexes and negatively interfere with their uptake by antigen presenting cells. These antagonists could be used to downregulate immune responses which are caused by cellular release of HSPs. Alternatively, certain  $\alpha$ 2MR HSP-binding domains may be used to enhance HSP-antigen complex uptake and signaling. Such agonists could be administered or expressed in subjects to elicit an immune response against an antigen of interest.

In another embodiment, the invention encompasses methods for identifying ligand fragment, such as HSP fragments, which are capable of binding and being taken up by the  $\alpha$ 2M receptor (" $\alpha$ 2M receptor-binding domains"), and analogs, muteins, or derivatives thereof. As described for assays for  $\alpha$ 2M receptor-related polypeptides described above, such  $\alpha$ 2M receptor-binding domains can then be tested for activity *in vivo* and *in vitro* using the binding assays described in Section 5.2.1, above. For example, one such method for identifying a heat shock protein fragment capable of binding an  $\alpha$ 2M receptor comprises: (a) contacting an  $\alpha$ 2M receptor with one or more heat shock protein fragments; and (b) identifying a heat shock protein fragment which specifically binds to the  $\alpha$ 2M receptor.

Ligand fragments, such as HSP fragments, of interest may be further tested in cells, using *in vivo* binding assays, re-presentation assays, or CTL assays, such as those described in Section 5.2.2, above. For example, in one embodiment, such a method for identifying a heat shock protein fragment capable of inducing an HSP- $\alpha$ 2M receptor-mediated process comprises: a) contacting an  $\alpha$ 2M receptor fragment with a cell expressing a heat shock protein; and b) measuring the level of  $\alpha$ 2MR activity in the cell, such that if the level of the HSP- $\alpha$ 2M receptor-mediated process or activity measured in (b) is greater than the level of  $\alpha$ 2MR activity in the absence of said heat shock protein fragment. Alternatively,  $\alpha$ 2M receptor-binding domains which decrease uptake of HSPs could be used to block HSP uptake by the  $\alpha$ 2M receptor. In one embodiment, such HSP fragments comprising  $\alpha$ 2M receptor-binding domain sequences could be used to construct recombinant fusion proteins, comprised of a heat shock protein  $\alpha$ 2M receptor-binding domain and an antigenic peptide sequence. Such recombinant fusion proteins may be used to elicit an immune response and to treat or prevent immune diseases and disorders (Suzue *et al.*, 1997, Proc. Natl. Acad. Sci. U.S.A. 94: 13146-51).

The  $\alpha$ 2M receptor fragments, analogs, muteins, and derivatives and/or ligand (*e.g.*, HSP) fragments, analogs, muteins, and derivatives of the invention may be produced by recombinant DNA techniques, synthetic methods, or by enzymatic or chemical cleavage of native  $\alpha$ 2M receptor and/or ligands (*e.g.*, HSPs).

Any eukaryotic cell may serve as the nucleic acid source for obtaining the coding region of an  $\alpha$ 2M receptor or  $\alpha$ 2M receptor ligand (*e.g.*, HSP) gene. Nucleic acid sequences encoding ligand, *e.g.*, HSPs, and or the  $\alpha$ 2M receptor can be isolated from vertebrate, mammalian, as well as primate sources, including humans. Amino acid sequences and

nucleotide sequences of naturally occurring ligands, *e.g.*, HSPs, and  $\alpha$ 2M receptor are generally available in sequence databases, such as Genbank.

The DNA may be obtained by standard procedures known in the art by DNA amplification or molecular cloning directly from a tissue, cell culture, or cloned DNA (*e.g.*, a DNA "library"). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. In a preferred embodiment, DNA can be amplified from genomic or cDNA by polymerase chain reaction (PCR) amplification using primers designed from the known sequence of an  $\alpha$ 2M receptor ligand, *e.g.*, HSP,  $\alpha$ 2M, or other  $\alpha$ 2MR ligand. The polymerase chain reaction (PCR) is commonly used for obtaining genes or gene fragments of interest. For example, a nucleotide sequence encoding a fragment of any desired length can be generated using PCR primers that flank the nucleotide sequence encoding the peptide-binding domain. Alternatively, an  $\alpha$ 2MR ligand, *e.g.*, HSP,  $\alpha$ 2M, or other  $\alpha$ 2MR ligand receptor gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) if such sites are available, releasing a fragment of DNA encoding the peptide-binding domain. If convenient restriction sites are not available, they may be created in the appropriate positions by site-directed mutagenesis and/or DNA amplification methods known in the art (see, for example, Shankarappa *et al.*, 1992, PCR Method Appl. 1:277-278). The DNA fragment that encodes a fragment of the ligand (*e.g.*, HSP) or  $\alpha$ 2M receptor gene is then isolated, and ligated into an appropriate expression vector, care being taken to ensure that the proper translation reading frame is maintained. Alternatives to isolating the genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the ligand (*e.g.*, HSP) and/or  $\alpha$ 2M receptor.

Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, or for creating/deleting restriction sites to facilitate further manipulations. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., *et al.*, 1978, J. Biol. Chem 253:6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19:423-463; Hill *et al.*, 1987, Methods Enzymol. 155:558-568), PCR-based overlap extension (Ho *et al.*, 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar *et al.*, 1990, Biotechniques, 8:404-407), etc. Modifications can be confirmed by double stranded dideoxy DNA sequencing.

An alternative to producing  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) fragments by recombinant techniques is peptide synthesis. For example, a peptide corresponding to a portion of an  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) comprising the substrate-binding domain, or which binds peptides *in vitro*, can be synthesized by use of a peptide synthesizer.



Conventional peptide synthesis may be used or other synthetic protocols well known in the art.

In addition, analogs and derivatives of  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) can be chemically synthesized. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino acids, designer amino acids such as  $\beta$ -methyl amino acids, C $\alpha$ -methyl amino acids, N $\alpha$ -methyl amino acids, and amino acid analogs in general.

$\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) peptides, or a mutant or derivative thereof, may be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, J. Am. Chem. Soc., 85:2149. During synthesis, N- $\alpha$ -protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric support *i.e.*, polystyrene beads. The peptides are synthesized by linking an amino group of an N- $\alpha$ -deprotected amino acid to an  $\alpha$ -carboxyl group of an N- $\alpha$ -protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N- $\alpha$ -protecting groups include Boc which is acid labile and Fmoc which is base labile. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (*See*, Atherton, *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, and Bodanszky, 1993, Peptide Chemistry, A Practical Textbook, 2nd Ed., Springer-Verlag).

Purification of the resulting fragment is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

In an alternative embodiment, fragments of an  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) may be obtained by chemical or enzymatic cleavage of native or recombinant  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) molecules. Specific chemical cleavage can be performed by cyanogen bromide, NaBH<sub>4</sub>, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, *etc.*. Endoproteases that cleave at specific sites can also be used. Such proteases are known in the art, including, but not limited to, trypsin,  $\alpha$ -chymotrypsin, V8 protease, papain, and proteinase K (*see* Ausubel *et al.*, (eds.), in

“Current Protocols in Molecular Biology”, Greene Publishing Associates and Wiley Interscience, New York, 17.4.6-17.4.8). The  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) amino acid sequence of interest can be examined for the recognition sites of these proteases. An enzyme is chosen which can release a peptide-binding domain or peptide-binding fragment.

5 The  $\alpha$ 2M receptor and/or ligand (*e.g.*, HSP) molecule is then incubated with the protease, under conditions that allow digestion by the protease and release of the specifically designated peptide-binding fragments. Alternatively, such protease digestions can be carried out blindly, *i.e.*, not knowing which digestion product will contain the peptide-binding domain, using specific or general specificity proteases, such as proteinase K or pronase.

10 Once a fragment is prepared, the digestion products may be purified as described above, and subsequently tested for the ability to bind peptide or for immunogenicity. Methods for determining the immunogenicity of  $\alpha$ 2M receptor ligand (*e.g.*, HSP) complexes by cytotoxicity tests are described in Section 5.2.2.

#### 15 5.4 DRUG DESIGN

Upon identification of a compound that interacts with  $\alpha$ 2MR, or modulates the interaction of an  $\alpha$ 2M receptor ligand, such as an HSP, with the  $\alpha$ 2M receptor, such a compound can be further investigated to test for an ability to alter the immune response. In particular, for example, the compounds identified via the present methods can be further

20 tested *in vivo* in accepted animal models of HSP- $\alpha$ 2MR-mediated processes and HSP-  $\alpha$ 2MR related disorders, such as, *e.g.*, immune disorders, proliferative disorders, and infectious diseases.

Computer modeling and searching technologies permit identification of compounds, or the improvement of already identified compounds, which can modulate the interaction of

25 the  $\alpha$ 2M receptor with its ligand, *e.g.*, an HSP. Having identified such a compound or composition, the active sites or regions are identified. Such active sites might typically be ligand binding sites. The active site can be identified using methods known in the art including, for example, from the amino acid sequences of peptides, from the nucleotide sequences of nucleic acids, or from study of complexes of the relevant compound or

30 composition with its natural ligand. In the latter case, chemical or X-ray crystallographic methods can be used to find the active site by finding where on the factor the complexed ligand is found.

Next, the three dimensional geometric structure of the active site is determined. This can be done by known methods, including X-ray crystallography, which can determine a

35 complete molecular structure. On the other hand, solid or liquid phase NMR can be used to determine certain intra-molecular distances. Any other experimental method of structure

determination can be used to obtain partial or complete geometric structures. The geometric structures may be measured with a complexed ligand, natural or artificial, which may increase the accuracy of the active site structure determined.

5 If an incomplete or insufficiently accurate structure is determined, the methods of computer based numerical modeling can be used to complete the structure or improve its accuracy. Any recognized modeling method may be used, including parameterized models specific to particular biopolymers such as proteins or nucleic acids, molecular dynamics models based on computing molecular motions, statistical mechanics models based on thermal ensembles, or combined models. For most types of models, standard molecular  
10 force fields, representing the forces between constituent atoms and groups, are necessary, and can be selected from force fields known in physical chemistry. The incomplete or less accurate experimental structures can serve as constraints on the complete and more accurate structures computed by these modeling methods.

Finally, having determined the structure of the active site, either experimentally, by  
15 modeling, or by a combination, candidate modulating compounds can be identified by searching databases containing compounds along with information on their molecular structure. Such a search seeks compounds having structures that match the determined active site structure and that interact with the groups defining the active site. Such a search can be manual, but is preferably computer assisted. These compounds found from this search are  
20 potential the  $\alpha$ 2M receptor-modulating compounds.

Alternatively, these methods can be used to identify improved modulating compounds from an already known modulating compound or ligand. The composition of the known compound can be modified and the structural effects of modification can be determined using the experimental and computer modeling methods described above applied  
25 to the new composition. The altered structure is then compared to the active site structure of the compound to determine if an improved fit or interaction results. In this manner systematic variations in composition, such as by varying side groups, can be quickly evaluated to obtain modified modulating compounds or ligands of improved specificity or activity.

30 Further experimental and computer modeling methods useful to identify modulating compounds based upon identification of the active sites of either the  $\alpha$ 2M receptor or the HSP, and other  $\alpha$ 2M receptor ligands and their analogs, will be apparent to those of skill in the art.

Examples of molecular modeling systems are the CHARMM and QUANTA  
35 programs (Polygen Corporation, Waltham, MA). CHARMM performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive

construction, modification, visualization, and analysis of the behavior of molecules with each other.

A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen *et al.*) 1988, Acta Pharmaceutica Fennica 97:159-166); Ripka  
5 (1988 New Scientist 54-57); McKinaly and Rossmann (1989, Annu. Rev. Pharmacol. Toxicol. 29:111-122); Perry and Davies, OSAR: Quantitative Structure-Activity Relationships in Drug Design pp. 189-193 Alan R. Liss, Inc. 1989; Lewis and Dean (1989, Proc. R. Soc. Lond. 236:125-140 and 141-162); and, with respect to a model receptor for nucleic acid components, Askew *et al.* (1989, J. Am. Chem. Soc. 111:1082-1090). Other  
10 computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc. (Pasadena, CA.), Allelix, Inc. (Mississauga, Ontario, Canada), and Hypercube, Inc. (Cambridge, Ontario). Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

## 15 5.5 DIAGNOSTIC USES

The  $\alpha 2M$  receptor is a cell surface protein present on many tissues and cell types (Herz *et al.*, 1988, EMBO J. 7:4119-27; Moestrup *et al.*, 1992, Cell Tissue Res. 269: 375-82), that appears to be involved in the specific uptake and re-presentation of  $\alpha 2M$  receptor  
20 ligands, such as HSPs and HSP-peptide complexes. The  $\alpha 2M$  receptor was initially identified as a heat shock protein receptor due to its interaction with gp96, which is exclusively intracellular and is released as a result of necrotic but not apoptotic cell death. Thus, gp96 uptake by the  $\alpha 2M$  receptor may act as a sensor of necrotic cell death. As such,  $\alpha 2M$  receptor-ligand complexes may be used to detect and diagnose proliferative disorders,  
25 such as cancer, autoimmune disorders and infectious disease. Therefore,  $\alpha 2M$  receptor proteins, analogues, derivatives, and subsequences thereof,  $\alpha 2M$  receptor nucleic acids (and sequences complementary thereto), and anti- $\alpha 2M$  receptor antibodies, have uses in detecting and diagnosing such disorders.

The  $\alpha 2M$  receptor and  $\alpha 2M$  receptor nucleic acids can be used in assays to detect,  
30 prognose, or diagnose immune system disorders that may result in tumorigenesis, carcinomas, adenomas etc, and viral disease.

The molecules of the present invention can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting  $\alpha 2M$  receptor expression, or monitor the treatment thereof. In particular, such an  
35 immunoassay is carried out by a method comprising contacting a sample derived from a patient with an HSP- $\alpha 2M$  receptor specific antibody under conditions such that

immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, in tissue sections, can be used to detect aberrant  $\alpha 2M$  receptor localization or aberrant (*e.g.*, low or absent) levels of  $\alpha 2M$  receptor. In a specific embodiment, antibody to the  $\alpha 2M$  receptor can be used to assay a patient tissue or serum sample for the presence of the  $\alpha 2M$  receptor where an aberrant level of  $\alpha 2M$  receptor is an indication of a diseased condition. By "aberrant levels," is meant increased or decreased levels relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disorder.

The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, immunohistochemistry radioimmunoassays, ELISA, "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

$\alpha 2M$  receptor genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays.  $\alpha 2M$  receptor nucleic acid sequences, or subsequences thereof, comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant changes in  $\alpha 2M$  receptor expression and/or activity as described *supra*. In particular, such a hybridization assay is carried out by a method comprising contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to  $\alpha 2M$  receptor DNA or RNA, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

In specific embodiments, diseases and disorders involving decreased immune responsiveness during an infection or malignant disorder can be diagnosed, or their suspected presence can be screened for, or a predisposition to develop such disorders can be detected, by detecting decreased levels of  $\alpha 2M$  receptor protein,  $\alpha 2M$  receptor RNA, or the  $\alpha 2M$  receptor functional activity (*e.g.*, binding to HSP, antibody-binding activity *etc.*), or by detecting mutations in  $\alpha 2M$  receptor RNA, DNA or  $\alpha 2M$  receptor protein (*e.g.*, translocations in the  $\alpha 2M$  receptor nucleic acids, truncations in the  $\alpha 2M$  receptor gene or protein, changes in nucleotide or amino acid sequence relative to wild-type  $\alpha 2M$  receptor) that cause decreased expression or activity of  $\alpha 2M$  receptor. Such diseases and disorders include but are not limited to those described in Sections 5.7, 5.8, and 5.9. By way of example, levels of the  $\alpha 2M$  receptor protein can be detected by immunoassay, levels of  $\alpha 2M$  receptor RNA can be detected by hybridization assays (*e.g.*, Northern blots, in situ-hybridization),  $\alpha 2M$  receptor activity can be assayed by measuring binding activities in vivo



or *in vitro*. Translocations, deletions, and point mutations in  $\alpha 2M$  receptor nucleic acids can be detected by Southern blotting, FISH, RFLP analysis, SSCP, PCR using primers, preferably primers that generate a fragment spanning at least most of the  $\alpha 2M$  receptor gene, sequencing of  $\alpha 2M$  receptor genomic DNA or cDNA obtained from the patient, *etc.*

5 In a preferred embodiment, levels of  $\alpha 2M$  receptor mRNA or protein in a patient sample are detected or measured relative to the levels present in an analogous sample from a subject not having the malignancy or hyperproliferative disorder. Decreased levels indicate that the subject may develop, or have a predisposition to developing, viral infection, malignancy, or hyperproliferative disorder.

10 In another specific embodiment, diseases and disorders involving a deficient immune responsiveness resulting in cell proliferation or in which cell proliferation is desirable for treatment, are diagnosed, or their suspected presence can be screened for, or a predisposition to develop such disorders can be detected, by detecting increased levels of the  $\alpha 2M$  receptor protein,  $\alpha 2M$  receptor RNA, or the  $\alpha 2M$  receptor functional activity (*e.g.*, HSP binding or  
15  $\alpha 2M$  receptor antibody, *etc.*), or by detecting mutations in  $\alpha 2M$  receptor RNA, DNA or protein (*e.g.*, translocations in  $\alpha 2M$  receptor nucleic acids, truncations in the gene or protein, changes in nucleotide or amino acid sequence relative to wild-type  $\alpha 2M$  receptor) that cause increased expression or activity of the  $\alpha 2M$  receptor. Such diseases and disorders include, but are not limited to, those described in Sections 5.7, 5.8, and 5.9. By way of example,  
20 levels of the  $\alpha 2M$  receptor protein, levels of  $\alpha 2M$  receptor RNA,  $\alpha 2M$  receptor binding activity, and the presence of translocations or point mutations can be determined as described above.

In a specific embodiment, levels of  $\alpha 2M$  receptor mRNA or protein in a patient sample are detected or measured, relative to the levels present in an analogous sample from a  
25 subject not having the disorder, in which increased levels indicate that the subject has, or has a predisposition to, an autoimmune disorder.

Kits for diagnostic use are also provided, that comprise in one or more containers an anti- $\alpha 2M$  receptor antibody, and, optionally, a labeled binding partner to the antibody. Alternatively, the anti- $\alpha 2M$  receptor antibody can be labeled (with a detectable marker, *e.g.*,  
30 a chemiluminescent, enzymatic, fluorescent, or radioactive moiety). A kit is also provided that comprises in one or more containers a nucleic acid probe capable of hybridizing to  $\alpha 2M$  receptor RNA. In a specific embodiment, a kit can comprise in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides) that are capable of priming amplification [*e.g.*, by polymerase chain reaction (see *e.g.*, Innis *et al.*, 1990, PCR Protocols,  
35 Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Q $\beta$  replicase, cyclic probe reaction, or other methods known in the art] under appropriate reaction conditions of at least a portion of an  $\alpha 2M$  receptor nucleic acid. A kit can optionally

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further comprise in a container a predetermined amount of a purified  $\alpha$ 2M receptor protein or nucleic acid, *e.g.*, for use as a standard or control.

## 5.6 THERAPEUTIC USES

5 The invention further encompasses methods for modulating the immune response. The  $\alpha$ 2M receptor recognizes and transports antigenic peptide complexes (*e.g.*, HSP-antigenic peptide complexes) for the purpose of presenting such antigenic molecules to cells of the immune system and eliciting an immune response. Thus, the compositions and methods of the invention may be used for therapeutic treatment of HSP- $\alpha$ 2M receptor-related disorders and conditions, such as autoimmune diseases, cancer and infectious diseases. In particular, as described in detail hereinbelow, recombinant cells comprising  $\alpha$ 2M receptor complexes, such as HSP-antigenic peptide complexes, antibodies and other compounds that interact with the  $\alpha$ 2M receptor, or modulate the interaction between the  $\alpha$ 2M receptor and its ligands, *e.g.*, HSP, as well as other compounds that modulate HSP- $\alpha$ 2M receptor-mediated processes may be used to elicit, or block, an immune response to treat such HSP-  $\alpha$ 2M receptor-related disorders and conditions.

### 5.6.1 THERAPEUTIC USE OF IDENTIFIED AGONISTS AND ANTAGONISTS

20 Compounds, such as those identified by screening methods provided herein, that interact with the  $\alpha$ 2M receptor (herein " $\alpha$ 2MR"), or modulate the interaction between the  $\alpha$ 2M receptor and its ligand, *e.g.*, HSP, can be useful as therapeutics. Such compounds, include, but are not limited to, agonists, antagonists, such as antibodies, antisense RNAs and ribozymes. Compounds which interfere with ligand (*e.g.*, HSP) - $\alpha$ 2M receptor interaction can be used to block an immune response, and can be used to treat autoimmune responses and conditions. Other antibodies, agonists, antagonists, antisense RNAs and ribozymes may upregulate ligand (*e.g.*, HSP)- $\alpha$ 2MR interaction, activity, or expression, and would enhance the uptake of antigen complexes (*e.g.*, HSP-antigen complexes), and therefore be useful in stimulating the host's immune system prior to, or concurrent with, the administration of a vaccine. Described below are methods and compositions for the use of such compounds in the treatment of HSP- $\alpha$ 2M receptor-related disorders, such as immune disorders, proliferative disorders, and infectious diseases.

35 In one embodiment an antagonist of  $\alpha$ 2M receptor-ligand (*e.g.*, HSP- $\alpha$ 2M receptor) interaction is used to block the immune response. Such antagonists include compounds that interfere with binding of a ligand (*e.g.*, an HSP) to the receptor by competing for binding to the  $\alpha$ 2M receptor, the ligand, or the ligand- $\alpha$ 2M receptor complex.

In one embodiment, the antagonist is an antibody specific for the  $\alpha$ 2M receptor, or a fragment thereof which contains the HSP ligand binding site. In another embodiment the antagonist is an antibody specific for an HSP, which interferes with binding of the HSP to the receptor.

5 In another embodiment, the antagonist is a peptide which comprises at least contiguous 10 amino acids of an HSP sequence. Such a peptide can bind to the ligand binding site of the  $\alpha$ 2M receptor and block the interaction of an HSP or HSP complex. In another embodiment, the antagonist is a peptide which comprises at least contiguous 10 amino acids of  $\alpha$ 2M sequence, which, like an HSP, can bind to the  $\alpha$ 2M receptor and  
10 interfere with the binding and uptake of HSP-antigen complexes. In yet another embodiment, the antagonist is a peptide which comprises at least contiguous 10 amino acids of  $\alpha$ 2M receptor sequence, in particular the ECD of the  $\alpha$ 2M receptor (or a portion thereof), which can bind to and "neutralize" natural ligands, such as HSPs,  $\alpha$ 2M, LDL, *etc.*

Such peptides may be produced synthetically or by using standard molecular biology  
15 techniques. Amino acid sequences and nucleotide sequences of naturally occurring  $\alpha$ 2M receptor ligands, such as  $\alpha$ 2M and HSPs, are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. Methods for recombinant and synthetic production of such peptides are described in Sections  
20 5.1.1 and 5.1.2.

Additionally, compounds, such as those identified via techniques such as those described hereinabove, in Section 5.2, that are capable of modulating  $\alpha$ 2M receptor gene product activity can be administered using standard techniques that are well known to those of skill in the art.

25

#### 5.6.1.1 COMPETITIVE ANTAGONISTS OF $\alpha$ 2MR-LIGAND INTERACTIONS

In one embodiment an antagonist of an  $\alpha$ 2Mr-ligand (*e.g.*, HSP-  $\alpha$ 2M receptor) interaction is used to block the immune response to an antigen complex, *e.g.*, to treat an auto-  
30 immune disorder. Such antagonists include molecules that interfere with binding by binding to the  $\alpha$ 2M receptor, thereby interfering with binding of a ligand (*e.g.*, HSP) to the receptor. An example of this type of competitive inhibitor is an antibody to  $\alpha$ 2M receptor, or a fragment of  $\alpha$ 2MR which contains an HSP ligand binding site. Another example of a competitive antagonist is  $\alpha$ 2M, or a receptor-binding fragment thereof, which itself binds to  
35  $\alpha$ 2MR, thereby blocking the binding and uptake of HSP-antigen complexes by the cell.

An  $\alpha$ 2MR-ligand (*e.g.*, HSP) competitive inhibitor can be any type of molecule, including but not limited to a protein, nucleic acid or drug. In a preferred embodiment, an

HSP- $\alpha$ 2M competitive inhibitor is an  $\alpha$ 2MR-binding or an HSP-binding peptide. Examples of such peptides are provided below.

#### 5.6.1.1.1 $\alpha$ 2M RECEPTOR-BINDING PEPTIDES

##### 5 $\alpha$ Macroglobulin peptides

In one embodiment of the present invention, an HSP- $\alpha$ 2MR competitive antagonist is an  $\alpha$  macroglobulin, preferably  $\alpha$ 2M, or  $\alpha$ 2MR-binding portion thereof.

Functional expression of  $\alpha$ 2M or  $\alpha$ 2MR-binding portions thereof (including recombinant expression as a FX fusion protein, processing, purification and refolding) is preferably carried out as described by Holtet *et al.*, 1994, FEBS Lett. 344:242-246.

In a specific mode of the embodiment, an  $\alpha$ 2MR-binding portion of  $\alpha$ 2M consists of or comprises a fragment of the  $\alpha$ 2M RBD consisting of at least 10 (continuous) amino acids. In other modes of the embodiment, the fragment consists of at least 20, 30, 40, 50, 75 or 100 amino acids of the RBD. In specific modes of the embodiment, such fragments are not larger than 27, 138 or 153 amino acids. Most preferred peptides comprise one or both of amino acids Lys<sub>1370</sub> and Lys<sub>1374</sub>. Such peptides include those consisting of amino acids 1299-1451 (vRBD in FIG. 13B) (SEQ ID NO:8), 1314-1451 (SEQ ID NO:9) (RBD in FIG. 13B) or 1366-1392 (SEQ ID NO:10) of the mature  $\alpha$ 2M protein. Other preferred peptides include but are not limited to those consisting of amino acids 1300-1425 (SEQ ID NO:11), 1300-1400 (SEQ ID NO:12), 1300-1380 (SEQ ID NO:13), 1325-1425 (SEQ ID NO:14), 1325-1400 (SEQ ID NO:15), 1325-1380 (SEQ ID NO:16), 1350-1425 (SEQ ID NO:17), 1350-1400 (SEQ ID NO:18), or 1350-1380 (SEQ ID NO:19) of the mature human  $\alpha$ 2M protein.

Derivatives or analogs of  $\alpha$ 2M or  $\alpha$ 2MR-binding portions of  $\alpha$ 2M are also contemplated as competitive antagonists of HSP- $\alpha$ 2MR complexes. Such derivative or analogs include but are not limited to those molecules comprising regions that are substantially homologous to  $\alpha$ 2M, the  $\alpha$ 2M RBD or fragments thereof (*e.g.*, in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding  $\alpha$ 2M RBD sequence, under stringent, moderately stringent, or nonstringent conditions. In certain specific embodiments, an  $\alpha$ 2M derivative is a chimeric or fusion protein comprising an  $\alpha$ 2M protein or  $\alpha$ 2MR-binding portion thereof (preferably consisting of at least 10 amino acids of the  $\alpha$ 2M RBD comprising Lys<sub>1370</sub> and Lys<sub>1374</sub>) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein.

In particular,  $\alpha$ 2M derivatives can be made by altering  $\alpha$ 2M coding sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due

to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a  $\alpha$ 2M gene may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or  $\alpha$ 2MR-binding portions of  $\alpha$ 2M genes which are altered by the substitution of different  
5 codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the  $\alpha$ 2M derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or an  $\alpha$ 2MR-binding portion of the amino acid sequence of an  $\alpha$ 2M protein, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence  
10 resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine,  
15 valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

The  $\alpha$ 2M derivatives and analogs of the invention can be produced by various  
20 methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned  $\alpha$ 2M gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s),  
25 followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of  $\alpha$ 2M, care should be taken to ensure that the modified gene remains within the same translational reading frame as  $\alpha$ 2M, uninterrupted by translational stop signals, in the gene region where the desired  $\alpha$ 2M activity is encoded.

30 Manipulations of the  $\alpha$ 2M sequence may also be made at the protein level. Included within the scope of the invention are  $\alpha$ 2M protein fragments or other derivatives or analogs which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of  
35 numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain,



V8 protease, NaBH<sub>4</sub>; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives of  $\alpha 2M$  can be chemically synthesized. For example, an  $\alpha 2MR$ -binding portion of  $\alpha 2M$  can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the  $\alpha 2M$  sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino acids, designer amino acids such as  $\beta$ -methyl amino acids, C $\alpha$ -methyl amino acids, N $\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In other specific modes of the embodiment, an HSP- $\alpha 2MR$  competitive antagonist is another  $\alpha$  macroglobulin or  $\alpha 2MR$ -binding portion thereof, for example an  $\alpha$  macroglobulin RBD domain selected from Nielsen *et al.*, *supra*, Fig. 3, Group A.

#### *RAP*

In one embodiment of the present invention, an HSP- $\alpha 2MR$  competitive antagonist is  $\alpha 2MR$ -associated protein (RAP) (Genbank accession no. A39875) or an  $\alpha 2MR$ -binding portion thereof. In a specific mode of the embodiment, an  $\alpha 2MR$ -binding portion of RAP consists of or comprises a fragment of the RAP RBD consisting of at least 10 (continuous) amino acids. In other modes of the embodiment, the fragment consists of at least 20, 30, 40, 50, 75 or 100 amino acids of the RBD. In specific modes of the embodiment, such fragments are not larger than 28, 50 or 100 amino acids. In other specific modes of the embodiment, an  $\alpha 2MR$ -binding portion of RAP comprises an  $\alpha 2MR$ -binding portion of domain 1 or 3, *e.g.* as depicted in Nielsen *et al.*, *supra*, Fig. 3, Group D or E. Expression of recombinant RAP or an  $\alpha 2MR$ -binding portion thereof, *e.g.* domain 1 or 3, is preferably achieved as described by Andersen *et al.*, *supra*).

#### 5.6.1.1.2 HSP-BINDING PEPTIDES

##### *$\alpha 2MR$ peptides*

In one embodiment of the present invention, an HSP- $\alpha 2MR$  competitive antagonist is  $\alpha 2MR$  peptide, preferably a soluble peptide, that can bind to HSPs and therefore competitively inhibit HSP binding to the native receptor.

Functional expression of HSP-binding portions of  $\alpha$ 2MR is preferably carried out as described for the CR8 domain by Huang *et al.*, 1999, J. Biol. Chem 274:14130-14136. Briefly, to maintain proper folding, the protein is expressed as a GST fusion, expressed recombinantly, the GST portion cleaved, uncleaved protein removed on GSH-Sepharose, and  
5 cleaved protein refolded. Since the complement repeats bind to calcium, proper folding is assayed by measuring the binding of the refolded protein to calcium.

In a specific mode of the embodiment, an HSP-binding portion of  $\alpha$ 2MR consists of or comprises at least one complement repeat, most preferably selected from CR3-CR10. In another specific mode of the embodiment, an HSP-binding portion of  $\alpha$ 2MR comprises a  
10 cluster of complement repeats, most preferably C1-II. In other modes of the embodiment, the HSP-binding portion consists of at least 10, more preferably at least 20, yet more preferably at least 30, yet more preferably at least 40, and most preferably at least 80 (continuous) amino acids. In specific modes of the embodiment, such fragments are not larger than 40-45 amino acids. In other specific modes of the embodiment, such fragments are not larger than  
15 80-90 amino acids. Exemplary preferred peptides include but are not limited to those consisting of amino acids 25-68 (SEQ ID NO:20), 25-110 (SEQ ID NO:21), 68-110 (SEQ ID NO:22), 853-894 (SEQ ID NO:23), 853-934 (SEQ ID NO:24), 853-974 (SEQ ID NO:25), 853-1013 (SEQ ID NO:26), 853-1060 (SEQ ID NO:27), 853-1102 (SEQ ID NO:28), 853-1183 (SEQ ID NO:29), 895-934 (SEQ ID NO:30), 895-974 (SEQ ID NO:31), 895-1013  
20 (SEQ ID NO:32), 895-1060 (SEQ ID NO:33), 895-1102 (SEQ ID NO:34), 895-1183 (SEQ ID NO:35), 935-974 (SEQ ID NO:36), 935-1013 (SEQ ID NO:37), 935-1060 (SEQ ID NO:38), 935-1102 (SEQ ID NO:39), 935-1183 (SEQ ID NO:40), 975-1013 (SEQ ID NO:41), 975-1060 (SEQ ID NO:42), 975-1143 (SEQ ID NO:43), 975-1183 (SEQ ID NO:44), 1014-1060 (SEQ ID NO:45), 1014-1102 (SEQ ID NO:46), 1014-1183 (SEQ ID  
25 NO:47), 1061-1102 (SEQ ID NO:48), 1061-1143 (SEQ ID NO:49), 1061-1183 (SEQ ID NO:50), 1103-1143 (SEQ ID NO:51), 1103-1183 (SEQ ID NO:52), or 1144-1183 (SEQ ID NO:53) of human  $\alpha$ 2MR.

Derivatives or analogs of HSP-binding portions  $\alpha$ 2MR also contemplated as competitive antagonists of HSP- $\alpha$ 2MR complexes. Such derivative or analogs include but  
30 are not limited to those molecules comprising regions that are substantially homologous to the extracellular domain of  $\alpha$ 2MR or fragments thereof (*e.g.*, in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of  
35 hybridizing to a sequence encoding an  $\alpha$ 2MR HSP-binding sequence, under stringent, moderately stringent, or nonstringent conditions. In certain specific embodiments, an  $\alpha$ 2MR derivative is a chimeric or fusion protein comprising an HSP-binding portion of  $\alpha$ 2MR,

preferably consisting of at least one complement repeat of C1-II) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. Such a chimeric protein can be produced recombinantly as described above, by omitting the cleavage repurification steps.

5 Other HSP-binding  $\alpha$ 2MR derivatives can be made by altering  $\alpha$ 2MR coding sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as an HSP-binding  $\alpha$ 2MR gene or gene fragment may be used in the practice of the present invention. Selection of suitable  
10 alterations and production of HSP-binding  $\alpha$ 2MR derivatives can be made applying the same principles described above for  $\alpha$ 2M derivatives and using the general methods described in Sections 5.1.1 and 5.1.2.

#### *HSP peptides*

15 In another mode of the embodiment, the antagonist is an peptide which comprises at least contiguous 10 amino acids of an HSP sequence. Such a peptide can bind to the ligand binding site of the  $\alpha$ 2M receptor a block the interaction of an HSP or HSP complex.

Such peptides may be produced synthetically or by using standard molecular biology techniques. Amino acid sequences and nucleotide sequences of naturally occurring HSPs are  
20 generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. Methods for recombinant and synthetic production of such peptides are described in Sections 5.1.1 and 5.1.2.

Additionally, compounds, such as those identified via techniques such as those  
25 described hereinabove, in Section 5.2, that are capable of modulating  $\alpha$ 2M receptor gene product activity can be administered using standard techniques that are well known to those of skill in the art.

#### 5.6.2 THERAPEUTIC USE OF THE $\alpha$ 2M RECEPTOR AGAINST CANCER AND INFECTIOUS DISEASES

30

In another embodiment, symptoms of certain  $\alpha$ 2M receptor gene disorders, such as autoimmune disorders, or proliferative or differentiative disorders causing tumorigenesis or cancer, may be ameliorated by modulating the level of  $\alpha$ 2M receptor gene expression and/or  $\alpha$ 2M receptor gene product activity. In one embodiment, for example, a decrease in  $\alpha$ 2M  
35 receptor gene expression may be useful to decrease  $\alpha$ 2M receptor activity, and ameliorate the symptoms of an autoimmune disorder. In this case, the level of  $\alpha$ 2M receptor gene expression may be decreased by using  $\alpha$ 2M receptor gene sequences in conjunction with

well-known antisense, gene "knock-out," ribozyme and/or triple helix methods. In another embodiment, an increase in  $\alpha$ 2M receptor gene expression may be desired to compensate for a mutant or impaired gene in an HSP- $\alpha$ 2M receptor-mediated pathway, and to ameliorate the symptoms of an HSP-  $\alpha$ 2M receptor-related disorder.

5 Among the compounds that may exhibit the ability to modulate the activity, expression or synthesis of the  $\alpha$ 2M receptor gene, including the ability to ameliorate the symptoms of an HSP- $\alpha$ 2M receptor related disorder are antisense, ribozyme, and triple helix molecules. Such molecules may be designed to reduce or inhibit either unimpaired, or if appropriate, mutant target gene activity. Techniques for the production and use of such  
10 molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. Antisense approaches involve the design of oligonucleotides that are complementary to a target gene mRNA. The antisense oligonucleotides will bind to the complementary target gene mRNA transcripts and  
15 prevent translation. Absolute complementarity, although preferred, is not required.

A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to  
20 hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

25 In one embodiment, oligonucleotides complementary to non-coding regions of the  $\alpha$ 2M receptor gene could be used in an antisense approach to inhibit translation of endogenous  $\alpha$ 2M receptor mRNA. Antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17  
30 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

In an embodiment of the present invention, oligonucleotides complementary to the nucleic acids encoding the HSP receptor ligand binding domain are used.

Regardless of the choice of target sequence, it is preferred that in vitro studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene  
35 expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control

RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.*, 1989, Proc. Natl. Acad. Sci. U.S.A. 86, 6553-6556; Lemaitre *et al.*, 1987, Proc. Natl. Acad. Sci. 84, 648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.*, 1988, BioTechniques 6, 958-976) or intercalating agents (see, *e.g.*, Zon, 1988, Pharm. Res. 5, 539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate (S-



ODNs), a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an  $\alpha$ -anomeric oligonucleotide. An  $\alpha$ -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gautier *et al.*, 1987, Nucl. Acids Res. 15, 6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue *et al.*, 1987, Nucl. Acids Res. 15, 6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, 1987, FEBS Lett. 215, 327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (1988, Nucl. Acids Res. 16, 3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. 85, 7448-7451), etc.

While antisense nucleotides complementary to the target gene coding region sequence could be used, those complementary to the transcribed, untranslated region are most preferred.

In one embodiment of the present invention, gene expression downregulation is achieved because specific target mRNAs are digested by RNase H after they have hybridized with the antisense phosphorothioate oligonucleotides (S-ODNs). Since no rules exist to predict which antisense S-ODNs will be more successful, the best strategy is completely empirical and consists of trying several antisense S-ODNs. Antisense phosphorothioate oligonucleotides (S-ODNs) will be designed to target specific regions of mRNAs of interest. Control S-ODNs consisting of scrambled sequences of the antisense S-ODNs will also be designed to assure identical nucleotide content and minimize differences potentially attributable to nucleic acid content. All S-ODNs can be synthesized by Oligos Etc. (Wilsonville, OR). In order to test the effectiveness of the antisense molecules when applied to cells in culture, such as assays for research purposes or *ex vivo* gene therapy protocols, cells will be grown to 60-80% confluence on 100 mm tissue culture plates, rinsed with PBS and overlaid with lipofection mix consisting of 8 ml Opti-MEM, 52.8  $\mu$ l Lipofectin, and a final concentration of 200 nM S-ODNs. Lipofections will be carried out using Lipofectin Reagent and Opti-MEM (Gibco BRL). Cells will be incubated in the presence of the lipofection mix for 5 hours. Following incubation the medium will be replaced with complete DMEM. Cells will be harvested at different time points post-lipofection and protein levels will be analyzed by Western blot.

Antisense molecules should be targeted to cells that express the target gene, either directly to the subject *in vivo* or to cells in culture, such as in ex vivo gene therapy protocols. A number of methods have been developed for delivering antisense DNA or RNA to cells; *e.g.*, antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

However, it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous target gene transcripts and thereby prevent translation of the target gene mRNA. For example, a vector can be introduced *e.g.*, such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290, 304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, 1980, Cell 22, 787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78, 1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296, 39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site. Alternatively, viral vectors can be used that selectively infect the desired tissue, in which case administration may be accomplished by another route (*e.g.*, systemically).

Ribozyme molecules designed to catalytically cleave target gene mRNA transcripts can also be used to prevent translation of target gene mRNA and, therefore, expression of target gene product. (See, *e.g.*, PCT International Publication WO90/11364, published October 4, 1990; Sarver *et al.*, 1990, Science 247, 1222-1225). In an embodiment of the present invention, oligonucleotides which hybridize to the HSP receptor gene are designed to be complementary to the nucleic acids encoding the HSP receptor ligand binding domain.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, Current Biology 4, 469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy target gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, Molecular Biology and Biotechnology: A Comprehensive Desk Reference, VCH Publishers, New York, (see especially fig. 4, p. 833) and in Haseloff & Gerlach, 1988, Nature, 334, 585-591, which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target gene mRNA, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug *et al.*, 1984, Science, 224, 574-578; Zaug and Cech, 1986, Science, 231, 470-475; Zaug *et al.*, 1986, Nature, 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been & Cech, 1986, Cell, 47, 207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the target gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.) and should be delivered to cells that express the target gene *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous target gene messages and inhibit translation. Because ribozymes unlike

antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous target gene expression can also be reduced by inactivating or "knocking out" the target gene or its promoter using targeted homologous recombination (*e.g.*, see Smithies *et al.*, 1985, Nature 317, 230-234; Thomas & Capecchi, 1987, Cell 51, 503-512; Thompson *et al.*, 1989, Cell 5, 313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous target gene (either the coding regions or regulatory regions of the target gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas & Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (*i.e.*, the target gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the target gene in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6), 569-584; Helene *et al.*, 1992, Ann. N.Y. Acad. Sci., 660, 27-36; and Maher, 1992, Bioassays 14(12), 807-815).

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC<sup>+</sup> triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles that the possibility may arise wherein the concentration of normal target gene product present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of target gene activity are maintained, therefore, nucleic acid molecules that encode and express target gene polypeptides exhibiting normal target gene activity may, be introduced into cells via gene therapy methods such as those described, below, in Section 5.6.3 that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the target gene encodes an extracellular protein, it may be preferable to co-administer normal target gene protein in order to maintain the requisite level of target gene activity.

Anti-sense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

### 5.6.3 GENE REPLACEMENT THERAPY

With respect to an increase in the level of normal  $\alpha$ 2M receptor gene expression and/or  $\alpha$ 2M receptor gene product activity,  $\alpha$ 2M receptor gene nucleic acid sequences can, for example, be utilized for the treatment of immune disorders resulting in proliferative disorders such as cancer. Such treatment can be administered, for example, in the form of gene replacement therapy. Specifically, one or more copies of a normal  $\alpha$ 2M receptor gene or a portion of the  $\alpha$ 2M receptor gene that directs the production of an  $\alpha$ 2M receptor gene product exhibiting normal  $\alpha$ 2M receptor gene function, may be inserted into the appropriate



cells within a patient, using vectors that include, but are not limited to adenovirus, adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes.

Gene replacement therapy techniques should be capable of delivering  $\alpha 2M$  receptor gene sequences to cell types that express the HSP receptor within patients. Thus, in one embodiment, techniques that are well known to those of skill in the art (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988) can be used to enable  $\alpha 2M$  receptor gene sequences to be delivered to developing cells of the myeloid lineage, for example, to the bone marrow. In another specific embodiment, gene replacement can be accomplished using macrophages *in vitro*, and delivered to a patient using the techniques of adoptive immunotherapy.

In another embodiment, techniques for delivery involve direct administration of such  $\alpha 2M$  receptor gene sequences to the site of the cells in which the  $\alpha 2M$  receptor gene sequences are to be expressed, *e.g.*, directly at the site of the tumor.

Additional methods that may be utilized to increase the overall level of  $\alpha 2M$  receptor gene expression and/or  $\alpha 2M$  receptor gene product activity include the introduction of appropriate  $\alpha 2M$  receptor-expressing cells, preferably autologous cells, into a patient at positions and in numbers that are sufficient to ameliorate the symptoms of an  $\alpha 2M$  receptor disorder. Such cells may be either recombinant or non-recombinant.

Among the cells that can be administered to increase the overall level of  $\alpha 2M$  receptor gene expression in a patient are cells that normally express the  $\alpha 2M$  receptor gene.

Alternatively, cells, preferably autologous cells, can be engineered to express  $\alpha 2M$  receptor gene sequences, and may then be introduced into a patient in positions appropriate for the amelioration of the symptoms of an  $\alpha 2M$  receptor disorder or a proliferative or viral disease, *e.g.*, cancer and tumorigenesis. Alternately, cells that express an unimpaired  $\alpha 2M$  receptor gene and that are from a MHC matched individual can be utilized, and may include, for example, brain cells. The expression of the  $\alpha 2M$  receptor gene sequences is controlled by the appropriate gene regulatory sequences to allow such expression in the necessary cell types. Such gene regulatory sequences are well known to the skilled artisan. Such cell-based gene therapy techniques are well known to those skilled in the art, see, *e.g.*, Anderson, U.S. Patent No. 5,399,349.

When the cells to be administered are non-autologous cells, they can be administered using well known techniques that prevent a host immune response against the introduced cells from developing. For example, the cells may be introduced in an encapsulated form which, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

#### 5.6.4 DELIVERY OF SOLUBLE $\alpha$ 2M RECEPTOR POLYPEPTIDES

Genetically engineered cells that express soluble  $\alpha$ 2M receptor ECDs or fusion proteins, *e.g.*, fusion Ig molecules can be administered *in vivo* where they may function as "bioreactors" that deliver a supply of the soluble molecules. Such soluble  $\alpha$ 2M receptor polypeptides and fusion proteins, when expressed at appropriate concentrations, should neutralize or "mop up" HSPs or other native ligand for the  $\alpha$ 2M receptor, and thus act as inhibitors of  $\alpha$ 2M receptor activity and may therefore be used to treat HSP- $\alpha$ 2M receptor-related disorders and diseases, such as autoimmune disorders, proliferative disorders, and infectious diseases.

#### 5.6.5 DELIVERY OF DOMINANT NEGATIVE MUTANTS

In another embodiment of the invention, dominant negative mutants ("dominant negatives") may be used therapeutically to block the immune response to an HSP-antigen complex, *e.g.*, to treat an auto-immune disorder. In general, such dominant-negatives are mutants which, when expressed, interact with ligand (*i.e.*, HSP-antigenic molecule complex), but lack one or more functions, *i.e.* endocytotic functions and/or signaling functions, of normal  $\alpha$ 2MR. Such mutants interfere with the function of normal  $\alpha$ 2MR in the same cell or in a different cell, *e.g.* by titration of HSP-peptide complexes from the wild type receptor. Such a mutation, for example, can be one or more point mutation(s), a deletion, insertion, or other mutation in either the extracellular of the 515 kDa subunit, or the extracellular, transmembrane or intracellular domains of the 85 kDa subunit of the alpha(2) macroglobulin receptor (*see* Krieger and Herz, 1994, *Annu. Rev. Biochem* 63:601-637 for  $\alpha$ 2MR subunit configuration). However, in construction of dominant negative mutations in the either subunit, care should be taken to ensure that the cleavage domain (signaling cleavage between aas 3525 and 3526 of the precursor of  $\alpha$ 2MR) remains intact so that the 515 kDa subunit is processed and presented on the cell surface. Additionally, care should be taken to ensure that the domains by which the two subunits associate should also remain functional. For example, in a specific embodiment, the C-terminal intracellular domain of the 85 kDa subunit is truncated. In another embodiment, a point mutation on the N-terminal 515 kDa subunit blocks endocytosis but not ligand binding. In another embodiment, the N-terminal 515 kDa subunit is expressed as a fusion protein, wherein the C-terminus of said fusion protein is the transmembrane domain and optionally the intracellular domain, of another Type I single transmembrane receptor.

Expression of a such a dominant negative mutation in cell can block uptake of ligand by normal functional receptors in the same or neighboring cells by titrating out the amount of available ligand. Thus, a recombinant antigen presenting cell expressing such a dominant

negative can be used to titrate out HSP-antigenic molecule complexes when administered to a patient in need of treatment for an autoimmune disorder.

#### 5.6.6 EXTRACORPOREAL METHODS FOR MODULATING THE IMMUNE RESPONSE

5 The present invention also relates to methods for modulating an immune response in a patient by altering the levels  $\alpha$ 2M receptor ligand in the bloodstream using extracorporeal methods.  $\alpha$ 2M receptor acts as a heat shock protein receptor in  $\alpha$ 2M receptor-expressing cells, such as macrophages and dendritic cells. Binding of HSPs or HSP antigenic peptide complexes to such  $\alpha$ 2M receptor-expressing cells results in internalization of the HSP and  
10 the re-presentation of peptides chaperoned by the HSP. However, because  $\alpha$ 2M receptor has a diverse roles in different cell types and binds numerous non-HSP ligands, competition between  $\alpha$ 2M receptor ligands reduces the ability of HSPs and HSP complexes to access  $\alpha$ 2M receptor.

15 The Applicant has discovered that depleting the blood of non-HSP- $\alpha$ 2M receptor ligands and transfusing such  $\alpha$ 2M receptor-ligand-depleted blood into the bloodstream of a patient can be used to stimulate the immune response, perhaps by increasing access of HSP complexes to the  $\alpha$ 2M receptor. Alternatively, blood can be depleted of  $\alpha$ 2M receptor ligands, including HSPs, followed by the addition of HSPs or HSP antigenic peptide complexes to stimulate a specific immune response. Decreasing the levels of  $\alpha$ 2M receptor  
20 ligands can be used to enhance a desired immune response in patients, such as patients with cancer and infectious disease. Such methods for depletion of  $\alpha$ 2M receptor ligands to the bloodstream are described in detail below.

In various embodiments, extracorporeal procedures, such as transfusion and  
25 apheresis, may be used to stimulate an immune response by modulating  $\alpha$ 2M receptor ligand levels in a patient's circulation or alternatively, depleting  $\alpha$ 2M receptor ligands including HSPs from the blood, followed by the selective addition of specific HSPs or HSP antigenic peptide complexes to the blood. For example, in one embodiment, apheresis techniques coupled with affinity column technology, are used to remove  $\alpha$ 2M receptor ligand from a  
30 patients blood, followed by the return the ligand-depleted blood into circulation.

In another embodiment, apheresis techniques coupled with affinity chromatography techniques are used to remove  $\alpha$ 2M receptor ligand from a patient's blood followed by the selective addition of HSPs or HSP antigenic peptide complexes to the patient's blood, and return of the treated blood into the patient's circulation.

35 Extraction of blood can be performed either manually or by any one of the common automated, electronically controlled "apheresis" systems such as the Autopheresis-C.RTM. system (Baxter Healthcare Corporation, Fenwal Division, 1425 Lake Cook Road, Deerfield,

Ill. 60015). In a preferred embodiment, a blood separation apparatus is fluidly connected to a blood vessel of the patient by way of a blood extraction tube. A blood pump, such as a peristaltic pump, is positioned on the blood extraction tube to pump blood from the patient to a blood separation apparatus. An anticoagulant, such as heparin, can be added to the blood through a separate chamber that is in fluid communication with the apheresis system.

Optionally, blood can be taken out of the apheresis system, treated to remove a  $\alpha$ 2M receptor ligand in the laboratory, and then put back into the apheresis system to be reintroduced to the patient. In another embodiment, the blood can be further separated into cellular components such that only a specific subset of cells (*i.e.* leukocytes) can be treated to remove an  $\alpha$ 2M receptor ligand and returned to the patient or, alternatively, only the plasma can be treated to remove an  $\alpha$ 2M receptor ligand and returned to the patient. In another embodiment, after the blood has been treated to remove an  $\alpha$ 2M receptor ligand, HSPs are added back to the blood.

In various embodiments, blood from a patient can be withdrawn manually and the cells can be separated by a standard laboratory blood cell collection device. After or during the cellular collection, the blood can be treated to remove an  $\alpha$ 2M receptor ligand. The cells can then be returned to the patient by an i.v. drip or by injection with a syringe.

In one embodiment, transfusion/apheresis methods may be used to enhance an immune response.  $\alpha$ 2M receptor ligands are removed from transfused blood of a patient in need of treatment for an immune disorder. In another embodiment, the  $\alpha$ 2M receptor ligand that is removed from the blood is not a heat shock protein.

One example of such a method comprises the following steps: (1) withdrawing blood from a patient; (2) passing the patient's blood over an affinity column comprising a  $\alpha$ 2M receptor ligand-binding compound, such as an antibody specific for a  $\alpha$ 2M receptor ligand, for a time period and under conditions sufficient to allow binding of  $\alpha$ 2M receptor ligand to the affinity column; (3) returning the  $\alpha$ 2M receptor-ligand depleted blood to the patient.

In another embodiment, apheresis methods may be used to enhance an immune response by depleting  $\alpha$ 2M receptor ligands (including HSPs) followed by the addition of selective HSPs or HSP antigenic peptide complexes to the blood of a patient.

An example of such a method comprises the following steps: (1) withdrawing blood from a patient; (2) passing the patient's blood over an affinity column comprising a  $\alpha$ 2M receptor-ligand-binding compound for a time period and under conditions sufficient to allow binding of the  $\alpha$ 2M receptor ligand to the affinity column; (3) adding HSPs or HSP antigenic peptide complexes to the ligand depleted blood; (4) returning the blood to the patient.

Methods that can be used to remove a ligand from the blood include affinity chromatography, anion or cation exchange chromatography, phosphocellulose chromatography, immunoaffinity chromatography, hydroxyapatite chromatography, and

lectin chromatography. Affinity purification is based on the interaction between the compound on the affinity column and its binding partner. The principle of affinity chromatography is well known in the art. In one embodiment, a recombinantly expressed and purified (or partially purified) protein, such as  $\alpha 2M$  receptor, is covalently or non-covalently coupled to a solid support such as, for example, a chromatography column. The extracted blood from a patient can be run over such a column. The coupled protein will bind to the  $\alpha 2M$  receptor ligand and deplete the blood of the  $\alpha 2M$  receptor ligand. The depleted blood can then be returned to the patient. In another embodiment, an antibody specific to the ligand can be coupled to the chromatography column and the immunospecific binding of an antibody to the  $\alpha 2M$  receptor ligand can be used to deplete the blood of the  $\alpha 2M$  receptor ligand. Alternatively, one of the many cation or anion exchange resins commonly used in the art can be used to deplete the blood of the  $\alpha 2M$  receptor ligand.

In another embodiment, the present invention also includes a kit that comprises a solid phase chromatography column with a purified  $\alpha 2M$  receptor ligand binding molecule attached thereto. Such a kit can contain components necessary for extracorporeal removal of  $\alpha 2M$  receptor ligands from the blood of a patient in need of such treatment.

Transfusion/apheresis methods may also be used in combination with other methods of immunotherapy. In one embodiment, for example, after depletion of non-HSP  $\alpha 2M$  receptor ligands as described above, HSP-antigenic peptide complexes may be delivered to a cancer patient, or a patient having an infectious disease, using the transfusion/apheresis methods, or other method. Using transfusion/apheresis, at the same time as HSP-antigenic peptide complexes are being delivered,  $\alpha 2M$  receptor ligands (other than HSPs) may be removed from the patient's blood, in order to stimulate the immune response against the HSP-antigenic peptide complex being delivered. Thus, the transfusion/apheresis method makes it possible to accomplish both the delivery of HSP-antigenic peptide complexes and the removal of competing  $\alpha 2M$  receptor ligands in a single procedure.

## 5.7 TARGET AUTOIMMUNE DISEASES

Autoimmune diseases that can be treated by the methods of the present invention include, but are not limited to, insulin dependent diabetes mellitus (*i.e.*, IDDM, or autoimmune diabetes), multiple sclerosis, systemic lupus erythematosus, Sjogren's syndrome, scleroderma, polymyositis, chronic active hepatitis, mixed connective tissue disease, primary biliary cirrhosis, pernicious anemia, autoimmune thyroiditis, idiopathic Addison's disease, vitiligo, gluten-sensitive enteropathy, Graves' disease, myasthenia gravis, autoimmune neutropenia, idiopathic thrombocytopenia purpura, rheumatoid arthritis, cirrhosis, pemphigus vulgaris, autoimmune infertility, Goodpasture's disease, bullous



pemphigoid, discoid lupus, ulcerative colitis, and dense deposit disease. The diseases set forth above, as referred to herein, include those exhibited by animal models for such diseases, such as, for example non-obese diabetic (NOD) mice for IDDM and experimental autoimmune encephalomyelitis (EAE) mice for multiple sclerosis.

5           The methods of the present invention can be used to treat such autoimmune diseases by reducing or eliminating the immune response to the patient's own (self) tissue, or, alternatively, by reducing or eliminating a pre-existing autoimmune response directed at tissues or organs transplanted to replace self tissues or organs damaged by the autoimmune response.

10

## 5.8 TARGET INFECTIOUS DISEASES

          The infectious diseases that can be treated or prevented using the methods and compositions of the present invention include those caused by intracellular pathogens such as viruses, bacteria, protozoans, and intracellular parasites. Viruses include, but are not limited  
15 to viral diseases such as those caused by hepatitis type B virus, parvoviruses, such as adeno-associated virus and cytomegalovirus, papovaviruses such as papilloma virus, polyoma viruses, and SV40, adenoviruses, herpes viruses such as herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), and Epstein-Barr virus, poxviruses, such as variola (smallpox) and vaccinia virus, RNA viruses, including but not limited to human  
20 immunodeficiency virus type I (HIV-I), human immunodeficiency virus type II (HIV-II), human T-cell lymphotropic virus type I (HTLV-I), and human T-cell lymphotropic virus type II (HTLV-II); influenza virus, measles virus, rabies virus, Sendai virus, picornaviruses such as poliomyelitis virus, coxsackieviruses, rhinoviruses, reoviruses, togaviruses such as rubella virus (German measles) and Semliki forest virus, arboviruses, and hepatitis type A  
25 virus.

          In another embodiment, bacterial infections can be treated or prevented such as, but not limited to disorders caused by pathogenic bacteria including, but not limited to, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Corynebacterium diphtheriae*, *Clostridium botulinum*, *Clostridium perfringens*,  
30 *Clostridium tetani*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis*, *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Campylobacter (Vibrio) fetus*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Bacillus cereus*, *Edwardsiella tarda*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*,  
35 *Salmonella typhimurium*, *Salmonella typhi*, *Treponema pallidum*, *Treponema pertenue*, *Treponema carateneum*, *Borrelia vincentii*, *Borrelia burgdorferi*, *Leptospira icterohemorrhagiae*, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Pneumocystis carinii*,

*Francisella tularensis*, *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Mycoplasma* spp., *Rickettsia prowazeki*, *Rickettsia tsutsugumushi*, *Chlamydia* spp., and *Helicobacter pylori*.

In another preferred embodiment, the methods can be used to treat or prevent infections caused by pathogenic protozoans such as, but not limited to, *Entamoeba histolytica*, *Trichomonas tenax*, *Trichomonas hominis*, *Trichomonas vaginalis*, *Trypanosoma gambiense*, *Trypanosoma rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, *Leishmania tropica*, *Leishmania braziliensis*, *Pneumocystis pneumonia*, *Plasmodium vivax*, *Plasmodium falciparum*, and *Plasmodium malaria*.

10

## 5.9 TARGET PROLIFERATIVE CELL DISORDERS

With respect to specific proliferative and oncogenic disease associated with HSP- $\alpha$ 2M receptor activity, the diseases that can be treated or prevented by the methods of the present invention include, but are not limited to: human sarcomas and carcinomas, e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, e.g., acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

Diseases and disorders involving a deficiency in cell proliferation or in which cell proliferation is desired for treatment or prevention, and that can be treated or prevented by inhibiting the  $\alpha$ 2M receptor function, include but are not limited to degenerative disorders, growth deficiencies, hypoproliferative disorders, physical trauma, lesions, and wounds; for

example, to promote wound healing, or to promote regeneration in degenerated, lesioned or injured tissues, etc.

## 5.10 PHARMACEUTICAL PREPARATIONS AND METHODS OF ADMINISTRATION

The compounds that are determined to affect  $\alpha 2M$  receptor gene expression or gene product activity can be administered to a patient at therapeutically effective doses to treat or ameliorate a cell proliferative disorder. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of such a disorder.

### 5.10.1 EFFECTIVE DOSE

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the  $LD_{50}$  (the dose lethal to 50% of the population) and the  $ED_{50}$  (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio  $LD_{50}/ED_{50}$ . Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the  $IC_{50}$  (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

### 5.10.2 FORMULATIONS AND USE

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

5 Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically  
10 acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well  
15 known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats);  
20 emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled  
25 release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from  
30 pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound  
35 and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit

dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

## 6. EXAMPLE: IDENTIFICATION OF $\alpha$ 2M RECEPTOR AS AN HSP RECEPTOR

### 6.1 INTRODUCTION

The Example presented herein describes the successful identification of an interaction between gp96, hsp90, hsp70, and calreticulin with the  $\alpha$ 2M receptor present in macrophages and dendritic cells. The experiments presented herein form the basis for isolating  $\alpha$ 2M receptor polypeptides and for the screening, diagnostic, and therapeutic methods of the present invention.

The Applicant of the present invention noted that certain observations were inconsistent with a "direct transfer" model of HSP-chaperoned peptide antigen presentation. First, the immunogenicity of HSP preparations is dependent on the presence of functional phagocytic cells but not B cells or other nonprofessional antigen-presenting cells, (Udono and Srivastava, 1993, *supra*; Suto and Srivastava, 1995, *supra*), whereas free peptides can sensitize all cell types. Second, extremely small quantities of HSP-peptide complexes were effective in eliciting specific immunity, *i.e.*, gp96-chaperoned peptides are several hundred times as effective as free peptides in sensitizing macrophages for CTL recognition,



suggesting the possibility of a specific uptake mechanism. Third, gp96-chaperoned peptides elicited an MHC I response that was not limited by the size of peptide. Finally, the processing of gp96-peptide complexes in macrophage was found to be sensitive to Brefeldin A (BFA), which blocks transport through the Golgi apparatus, suggesting that processing occurred through an intercellular mechanism. These observations led to the hypothesis that HSP-chaperoned peptides may be processed internally and re-presented by MHC class I molecules on the cell surfaces of macrophages (Suto and Srivastava, 1995, *supra*). There is also the hypothesis that the mannose receptor is used in the uptake of gp96 but no mechanism has been proposed for the non-glycosylated HSPs, such as HSP70 (Ciupitu *et al.*, 1998, J. Exp. Med., 187: 685-691). Others suggested that a novel intracellular trafficking pathway may be involved for the transport of peptides from the extracellular medium into the lumen of ER (Day *et al.*, 1997, Proc. Natl. Acad. Sci. 94:8065-8069; Nicchitta, 1998, Curr. Opin. in Immunol. 10:103-109). Further suggestions include the involvement of phagocytes which (a) possess an ill-defined pathway to shunt protein from the phagosome into the cytosol where it would enter the normal class I pathway; (b) digest ingested material in lysosomes and regurgitate peptides for loading on the surface to class I molecules (Bevan, 1995, J. Exp. Med. 192:639-41). The discovery of a receptor for heat shock proteins as disclosed herein helps to resolve the paradox of how extracellular antigenic peptides complexed to HSPs can be presented by MHC class I molecules on antigen presenting cells.

## 6.2 MATERIALS AND METHODS

*Mice, cells, and reagents.* C57Bl/6, BALB/c and TAP(-/-) mice were obtained from Jackson laboratories. Bone marrow-derived DCs were generated from the femurs and tibia of C57BL/6 mice. The bone marrow was flushed out and the leukocytes obtained and cultured as described (Lutz *et al.*, 1999, J. Immunol. Methods 223:77-92) in complete RPMI1640 with 10% heat inactivated FCS and 20ng/ml GMCSF (Endogen Inc., Woburn, MA) for 6 days. On day 3 fresh media with GMCSF was added to the plates for the day 6 cultures. Macrophages were obtained from PEMs of pristaned mice by positive selection for CD11b+ cells (Miltenyi Biotech Inc.). RAW264.7 was gift of Dr. Christopher Nicchitta. A20.25 was gift of Dr. Lawrence Kwak. All other cell lines were obtained from ATCC. Proteasome inhibitor Lactacystin was purchased from Kamiya Inc. Japan. Anti-CD91 antibody (clone 5A6) was purchased from PRAGEN (Heidelberg). Anti-hsp70 (clone N27F3) and anti-PDI (clone 1D3) antibodies were purchased from StressGen (Victoria, Canada).

*Purification of HSPs.* HSPs were purified as described (Srivastava, P.K., 1997, Methods: A companion to Methods in Enzymology 12:165-171; Basu and Srivastava, 1999, J. Exp. Med. 189(5):797-802). All buffers used for purifications were prepared with endotoxin free water (Nanopure Infinity UV/UF, Barnstead/Thermolyne, Dubuque, IA) and

all glasswares used for purification were cleaned with endotoxin free water and baked in a 4000F oven (Gruenberg, Williamsport, PA). The HSP-containing fractions were identified by immunoblots.

*Conjugation of proteins to FITC and staining of cells.* Purified proteins were  
5 conjugated to FITC using the FluoroTag FITC conjugation kits (SIGMA) as per the  
manufacturers protocol. Conjugation was confirmed by a 2kDa increase in molecular weight  
by SDS-PAGE and by immunoblotting with an anti-FITC monoclonal antibody. Incubations  
of indicated amounts of FITC-tagged proteins and cells were done in the presence of 1%  
nonfat dry milk (Carnation®) in PBS for 20min at 4°C. After repeated washing, cells were  
10 analyzed by flow cytometry (Becton Dickenson, La Jolla, California). Cells were also labeled  
with propidium iodide just before FACScan analysis. Cells staining positive for propidium  
iodide were gated out of the events. No differences were observed in the binding of HSPs to  
Mac-1+ cells from pristaned or non-pristaned mice. Fixed or unfixed cells were labeled with  
FITC-tagged HSP as above. Labeled cells were visualized using a Zeiss LSM confocal  
15 microscope.

*Affinity chromatography.* Proteins (1mg) in 2ml volume were incubated with 2ml of  
equilibrated AminoLink beads in PBS with a reductant (NaCNBH<sub>3</sub>) for 1 hour. Uncoupled  
protein was removed by extensive washing of the column and unreactive groups quenched.  
Immobilization yields were typically >92% of the starting amount of protein. Columns were  
20 stored at 4°C until used. Such columns were made with gp96 (purified as described in  
Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci., U.S.A. 83:3407-3411) and albumin. For  
membrane purification, cells were lysed by dounce homogenization in hypotonic buffer  
containing PMSF. Unlyzed cells and nuclei were removed by centrifugation at 1000g for 5  
mm. The postnuclear supernatant was centrifuged at 100,000g for 90 mins. The pellet  
25 contains total membranes and was fractionated by aqueous two-phase partition with a  
dextran/polyethylene glycol biphasic. Briefly membranes were resuspended in PEG (33%  
wt/wt in 0.22 M sodium phosphate buffer, pH 6.5) and underlaid gently with dextran  
(20%wt/wt in 0.22M sodium phosphate buffer, pH 6.5). The two phases were mixed gently  
and centrifuged at 2000 g for 15 mins. The white material at the interphase was enriched for  
30 plasma membranes, whose proteins were extracted by 2 hr incubation in 20mM Tris buffer  
(pH8, containing 0.08% octylglucoside) at 4°C.

*Photo cross-linking of gp96 to putative receptor.* The cross-linker (SASD, (Pierce)  
was labeled with I<sup>125</sup> using iodobeads (Pierce). Radiolabeled SASD was covalently attached  
to gp96 by incubation at room temperature for 1 hr. Free SASD and I<sup>125</sup> were removed by  
35 size exclusion column (KwikSep columns, Pierce). For cross-linking studies, I<sup>125</sup>-SASD-  
gp96 (50 µg gp96) was incubated with purified CD11b<sup>+</sup> cells. Unbound protein was  
removed by washing. All procedures to this point were carried out in very dim light.

Proteins were cross-linked with UV light. Cells were lysed with lysis buffer (0.5%NP4O, 10mM Tris, 1mMEDTA, 150mM NaCl) and treated with 100 mM 2-mercaptoethanol to cleave the cross-linker. *Cell lysates were analyzed by SDS-PAGE and autoradiography.*

*Re-presentation assays.* Re-presentation assays were carried out as described (Suto and Srivastava, 1995, Science 269:1585-1588). Antigen presenting cells (RAW264.7 macrophage cell line) were plated at a 1:1 ratio with AH I -specific T cells in complete RPMI. Approximately 10,000 cells of each type were used. Gp96 (10 µg/ml) chaperoning the AH1-20 mer peptide (RVTYHSPSYVYHQFERRAK) was added to the cells and the entire culture was incubated for 20 hrs. Stimulation of T cells was measured by quantifying the amount of IFN-γ released into the supernatants by ELISA (Endogen). In addition, CD11b+ peritoneal exudate cells (1X10<sup>4</sup>) were pulsed with HSPs purified from liver, or HSP-peptide complex generated in vitro and relevant CD8+ T cells (VSV8 specific CTL line or AH1-specific CTL clones, as indicated) were added to the cultures. The assay was carried out in 250 ml volume in 96-well plates with RPMI medium containing 5% FCS at 37°C for 20 hours. Culture supernatants were harvested and tested for the presence of IFN-γ release by ELISA (Endogen Inc., Woburn, MA).

*Complexing in vitro of peptide to HSPs.* HSPs were mixed with VSV19 or AH1-19 in a 50 : 1 peptide to protein molar ratio in 0.7M NaCl in Na - phosphate buffer and heated at 500 C for 10 min., then incubated at room temperature for 30 min. Excess free peptide was removed with PBS using centricon 10 ( Amicon, Inc., Beverly MA).

*Purification of CD11b+ cells.* CD11b+ cells were selected using the MACS columns and protocols supplied by Miltenyi Biotec Inc. Auburn, California. CD11b antibody, supplied as CD11b MicroBeads, was purchased from Miltenyi Biotec Inc., and has been demonstrated not to activate CD11b+ cells with regard to the markers tested in this experiment.

*Induction of cytotoxic T cells.* C57BL/6 mice were immunized intraperitoneally with 50 mg of gp96 complexed with VSV19 peptide. Ten days later, recipient spleens were removed and splenocytes were stimulated with VSV8 synthetic peptide at 1mM concentration. After 5 days, MLTCs were tested for cytotoxicity in a chromium release assay using EL4 cells alone and EL4 cells pulsed with VSV8 peptide as targets.

*Protein Microsequencing.* Proteins identified by affinity chromatography were analyzed on SDS-PAGE and stained with coomassie blue or transferred onto PVDF membrane and stained with coomassie blue, all of it under keratin-free conditions. Protein bands were excised with a razor from the gel or membrane. Tryptic peptides from an 80kDa faint coomassie band were extracted by 50% acetonitrile, 5% formic acid, dried, and loaded onto a 75 m 10 cm, reverse-phase C18, microcapillary column (3 µl vol) and tryptic peptides were separated by on-line microcapillary liquid chromatography-tandem mass spectrometry

followed by database searching using the SEQUEST program as previously described. (Gatlin *et al.*, 2000, Anal. Chem. 72:757-63; Link *et al.*, 1999, Nat. Biotechnol. 17:676-82). The analysis was carried out in a data-dependent auto-MS/MS fashion using a Finnigan LCQ iontrap Mass Spectrometer.

### 6.3 RESULTS

*Identification of an 80 kDa protein as a potential gp96 receptor.* Homogenous preparations of gp96 were coupled to FITC and the gp96-FITC was used to stain RAW264.7 cells, shown to be functionally capable of re-presenting gp96-chaperoned peptides. Gp96-FITC but not control albumin-FITC preparations stained the cell surface of RAW264.7 cells (FIG. 1A). Plasma membrane preparations of cell surface-biotinylated RAW264.7 cells were solubilized in 0.08% octyl-glucoside and the soluble extract was applied to a gp96-Sepharose column. The bound proteins were eluted with 3M sodium chloride. SDS-PAGE analysis of the eluate showed 2 major bands of ~75-80 kDa size (FIG. 1B, top left). Blotting of this gel with avidin-peroxidase showed that both bands were biotinylated, indicating their surface localization (FIG. 1B, bottom left). Affinity purification of membrane extracts of RAW264.7 cells over control serum albumin affinity columns did not result in isolation of any proteins, nor did probing of immunoblots of such gels with avidin peroxidase detect any albumin-binding surface proteins (FIG. 1B, top and bottom center lanes). As an additional control, chromatography of membrane extracts of P815 cells which do not bind gp96-FITC and which do not re-present gp96-chaperoned peptides, on gp96 affinity columns did not result in elution of any gp96-binding proteins (FIG. 1B, top and bottom right lanes).

In parallel experiments, gp96 molecules were coupled to the radio-iodinated linker sulfosuccinimidyl (4-azidosalicylamido) hexanoate (SASD) which contains a photo cross-linkable group. Gp96-SASD- $I^{125}$  was pulsed onto peritoneal macrophages, which have been shown previously to re-present gp96-chaperoned peptides (Suto and Srivastava, 1995, Science 269:1585-1588). Excess gp96-SASD was removed by multiple rounds of washing of the cells and photoactivation was carried out by exposure of cells to UV light for 10 min. Cell lysates were reduced in order to transfer the  $I^{125}$  group to the putative gp96 ligand and were analyzed by SDS- PAGE followed by autoradiography. The gp96 molecule was observed to cross-link to an ~80 kDa band specifically present in re-presentation-competent macrophage but not in the re-presentation-incompetent P815 cells (FIG. 1C). This band appears to correspond in size to the larger of the two bands seen in eluates of gp96 affinity columns (FIG. 1B). No band corresponding to the lower band in that preparation is seen in the photo cross-linked preparation. The observation of a specific binding of gp96 to an 80 kDa protein in two different re-presentation-competent cell types, but not in a re-



presentation-incompetent cell line, and by two independent assays supported the candidacy of the 80 kDa molecule for the gp96 receptor.

*Antiserum against the 80 kDa protein inhibits re-presentation of a gp96-chaperoned antigenic peptide.* The eluates containing the 75-80 kDa proteins were used to immunize a New Zealand white rabbit, and pre-immune and immune sera were used to probe blots of plasma membrane extracts of the re-presentation-competent RAW264.7 and primary peritoneal macrophages and the re-presentation-incompetent P815 cells. The immune but not the pre-immune serum detected the 80 kDa band (and a faint lower 75 kDa band) in plasma membrane extracts of primary macrophage and the RAW264.7 membranes but not of P815 cells (FIG. 2A). The pre-immune and immune sera were tested in a functional assay for their ability to block re-presentation of gp96-chaperoned peptides. The L<sup>d</sup>-restricted epitope AH1 derived from the gp70 antigen of murine colon carcinoma CT26 (Huang *et al.*, 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9730-9735) was used as the model system. Complexes of gp96 with an AH1 precursor (used to inhibit direct presentation) were pulsed onto RAW264.7 cells which were used to stimulate a L<sup>d</sup>/AH1-specific CD8<sup>+</sup> T cell clone. Release of interferon- $\gamma$  by the clones was measured as a marker of their activation. RAW264.7 cells were able to re-present gp96-chaperoned AH1 precursor effectively in this assay. It was observed that at the highest concentration, the immune sera inhibited re-presentation completely (FIG. 2B). Although the pre-immune serum was ineffective in inhibiting re-presentation as compared to the immune sera, it did inhibit re-presentation significantly at higher concentrations. The significance of this observation became clear later when we determined the identity of the gp96 receptor. Repeated immunizations with the affinity-purified gp96-binding proteins did not result in corresponding increase in antibody titers.

*Identification of the 80 kDa protein as an amino terminal fragment of the heavy chain of the  $\alpha$ 2M receptor.* The 80 kDa protein eluted from the gp96 affinity column was resolved on SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. The protein band was subjected to in-gel trypsin digestion and mass spectrometry-based protein microsequencing as described in the methods in Section 6.2. Four independent tryptic peptides corresponding to N-terminal region of the  $\alpha$  2-macroglobulin ( $\alpha$ 2M) receptor, designated by immunologists as CD91, were identified (FIG. 3C).

*$\alpha$ 2M inhibits re-presentation of a gp96-chaperoned antigenic peptide by RAW264.7.*  $\alpha$ 2M receptor is one of the known natural ligands for the  $\alpha$ 2M receptor. Its ability to inhibit re-presentation of gp96-chaperoned antigenic peptide AH1 was tested in the assay described in FIG. 2.  $\alpha$ 2M but not control proteins selectin (CD62) or serum albumin was observed to inhibit re-presentation completely and titratably (FIG. 4). This observation was also consistent with the result in FIG. 2 that while the pre-immune serum did not detect an 80 kDa band in plasma membranes of RAW264.7 cells, it did inhibit re-presentation to some



degree at high concentrations. Thus, by structural as well as functional criteria, the  $\alpha_2$ M receptor was determined to fulfill the criteria essential for a receptor for gp96.

*Binding of fluorescence-labeled HSPs and  $\alpha_2$ -macroglobulin to a panel of primary and cultured cells.* FITC-labeled HSPs, gp96, hsp90 or hsp70, or control non-HSP serum albumin (SA) were incubated with primary cells such as pristane-induced peritoneal macrophage, differentiated bone marrow-derived dendritic cells or with immortalized cell lines such as RAW264.7, RAW309Cr.1 of macrophage origin, P815 mastocytoma, YAC-1 lymphoma, EL4 thymoma, Meth A and PS-C3H fibrosarcomas, B16 melanoma, CT26 colon carcinoma, and UV6139 squamous cell carcinoma, as described in the Methods. After removal of unbound protein by extensive washing, cells were analyzed by flow cytometry. As shown in Figure 5, the peritoneal macrophages and the bone marrow-derived dendritic cells showed robust binding of each of the three HSPs but not albumin. However, of the two macrophage cell lines, only one of them, RAW264.7, bound the three HSPs. RAW309Cr.1 did not bind any of the HSPs (FIG. 6A and 6B). Out of 8 other cell lines tested with the FITC-labeled gp96, hsp90 and hsp70, none was observed to bind to HSP in a manner comparable to the binding observed with RAW264.7. YAC 1 was observed to bind hsp70 but only to a significantly smaller degree. The binding was only a fraction of that observed with APCs.

As described above, the  $\alpha_2$  macroglobulin receptor has been identified as the receptor for gp96. All of the cell types in Figure 5 were also tested for the presence of CD91 by staining with FITC- $\alpha_2$  macroglobulin. CD91 showed precisely the same pattern of distribution as did each of the three HSPs (FIG. 5).

*The ability of cells to bind HSPs and  $\alpha_2$ M correlates with the ability to re-present gp96-chaperoned peptides.* We tested if the ability of a particular cell type to bind HSPs or  $\alpha_2$  macroglobulin as shown in Figure 5 correlates with its ability to re-present gp96-chaperoned peptides. Re-presentation studies are done typically by incubating APCs and an HSP, chaperoning a known peptide, with T cells specific for an epitope present in the chaperoned peptide (Suto and Srivastava, 1995, *supra*). The experimental system is set up such that the peptide cannot charge directly onto MHC I but requires intracellular processing followed by presentation to T cells. VSV8 and AH1 antigenic systems were used in these studies. The VSV8 epitope (RGYVYQGL) is presented by the K<sup>b</sup> molecule and VSV19 (SLSDL RGYVYQGLKSGNVS) is its extended variant, which cannot charge K<sup>b</sup> directly. AH1 (SPSYVYHQF) is an L<sup>d</sup>-restricted epitope of a murine leukaemia virus envelope protein gp70 (Huang et al., 1996), and AH1-19 (RVTYHSPSYVYHQFERRAK) is its extended version. Peritoneal macrophage and BM-DCs were tested side-by-side for re-presentation in the VSV8 system, and both cell types were able to re-present gp96-

chaperoned VSV19 to VSV8-specific T cells (FIG. 7A). EL4 and B16 cells, both of the *b* haplotype, were also tested and were found unable to re-present in identical assays (data not shown). The BM-DCs were observed to re-present gp96-chaperoned VSV19 significantly better than macrophage did; however, it is not possible to determine from the data if this difference derives from the better T cell stimulatory properties of DCs in general or whether the DCs are specifically more efficient than macrophage at re-presenting gp96-chaperoned peptides. The two macrophage cell lines RAW309Cr.1 and RAW264.7 were tested for their re-presentation ability in the AH1 system. In parallel with the HSP and  $\alpha$ 2M-staining data (FIG. 5), RAW264.7 cells but not RAW309Cr.1 were observed to be capable of re-presenting gp96-chaperoned AH1 peptides (Fig. 7B).

*Peptides chaperoned by hsp90, hsp70 and CRT are re-presented by MHC I molecules of APCs.* Gp96 was the first HSP for which the re-presentation phenomenon was experimentally shown (Suto and Srivastava 1995, *supra*). Hsp70-chaperoned peptides have been shown recently to be re-presented by APCs (Castellino *et al.*, 2000, J.Exp Med. 191(11):1957-1964). The ability of other HSPs, hsp90 and CRT to introduce chaperoned peptides into the endogenous presentation pathway was tested in the AH1 system with RAW264.7 cells as the APCs. RAW264.7 cells were pulsed with hsp90, hsp70, calreticulin, or gp96, as a positive control, by themselves, or chaperoning the AH1-19 peptide. Chaperoning of peptides by the HSPs was accomplished in vitro as previously described (Blachere *et al.* 1997, J.Exp. Med. 186:1315-1322; Basu and Srivastava 1999, J. Exp. Med.189:797-802). T cells specific for L<sup>d</sup>/AH-1 secreted IFN- $\gamma$  when the RAW264.7 cells were pulsed with complexes of hsp90, hsp70, CRT or gp96 with AH1-19, but not when the HSPs were not complexed with the peptide (FIG. 8). Pulsing of RAW264.7 cells with AH1-19 alone did not lead to surface loading of L<sup>d</sup> molecules and consequent stimulation of T cells. Further, RAW264.7 cells pulsed with complexes of serum albumin with AH1-19, also failed to stimulate L<sup>d</sup>/AH1-specific T cells, thus indicating the specific requirement of HSPs for introducing the chaperoned peptides into the endogenous presentation pathway (FIG. 8).

*Gp96, hsp90, hsp70 and CRT engage a common receptor.* Does each HSP have a unique receptor or do they share a common receptor? This question was addressed by three independent criteria : by measuring re-presentation of gp96-chaperoned AH1-19 (as in FIGS. 7 and 8) in the presence of excess and titrated quantities of free (i.e. not complexed to AH1-19) gp96, hsp90, hsp70 or serum albumin, by testing if  $\alpha_2$  macroglobulin, a known ligand for CD91, a receptor for gp96, can inhibit re-presentation of peptides chaperoned by gp96, hsp90, hsp70 or CRT, and finally, if anti-CD91 antibody can inhibit re-presentation of peptides chaperoned by some or all the HSPs.

The gp96-AH1-19 complex was added to RAW264.7 cultures at a fixed final concentration of 40  $\mu\text{g/ml}$ , while the competing HSPs or serum albumin were added at concentrations between (200-800)  $\mu\text{g/ml}$ . It was observed (FIG. 9A) that all 3 competing HSPs could inhibit re-presentation of gp96-chaperoned AH1-19, albeit with different efficiencies. Gp96 was able to compete with itself, while hsp90 was an even better competitor than gp96. Hsp70 was a less efficient competitor than gp96 but was a significant competitor. Albumin competed inefficiently. In quantitative terms, approximately 2 fold molar excess of hsp90, 6 fold molar excess of gp96, and a 13 fold molar excess of hsp70 were required to inhibit by 50% the re-presentation of gp96-chaperoned peptides at a gp96 concentration of 40  $\mu\text{g/ml}$ . All three HSPs were able to inhibit the re-presentation of gp96-chaperoned peptides completely at the highest concentration tested. This observation suggests that gp96, hsp90 and hsp70 utilize a single receptor albeit with differing specificities.

In additional experiments, increasing quantities of  $\alpha_2$  macroglobulin were added to re-presentation assays where AH1-19 chaperoned by gp96, hsp90, hsp70 or CRT was re-presented by RAW264.7 cells, to L<sup>d</sup>/AH-1 specific T cells.  $\alpha_2$  macroglobulin was observed to inhibit, in a titratable manner, re-presentation of peptides chaperoned by each of the four HSPs (FIG. 9B). Re-presentation of peptides chaperoned by gp96, hsp70 and CRT was inhibited equally, while re-presentation of hsp90-chaperoned peptide was inhibited the most effectively, and almost completely at the highest concentration of  $\alpha_2$  macroglobulin tested. Serum albumin, when tested at the highest concentration, inhibited re-presentation only modestly. It may be noted that while the data in Fig. 9A show that the specific peptide-deficient HSPs inhibited re-presentation of gp96-AH1-19 complexes completely at the highest concentrations tested,  $\alpha_2$  macroglobulin appears far less effective, in quantitative terms, at inhibiting the re-presentation of peptides chaperoned by 3 of the 4 HSPs (FIG. 9B). However, this quantitative disparity disappears if one notes that the  $\alpha_2$  macroglobulin molecule is approximately 10 times larger in molecular mass than the average HSP molecule.

A mouse monoclonal anti-CD91 IgG<sub>1</sub> antibody and isotype control antibodies were tested for their ability to inhibit re-presentation of peptides chaperoned by gp96, hsp90, hsp70 and CRT. As before, the RAW264.7/AH1 system was utilized and the antibodies were added to re-presentation cultures at the concentrations indicated (Fig. 9C). Anti-CD91 antibody was observed to inhibit, titratably and specifically, the re-presentation of AH1 chaperoned by each of the 4 HSPs tested. The isotype control antibody did not inhibit re-presentation in any instance. Further, the inhibition mediated by the anti-CD91 antibody was complete and uniform for each of the HSPs, indicating that CD91 is the sole receptor for each of the 4 HSPs.

*Requirement of a functional proteasome complex for the representation of gp96-chaperoned peptides by APCs.* The re-representation assay was carried out in presence or absence of the specific proteasome inhibitor, lactacystin. The peritoneal macrophages were treated or untreated with lactacystin for 2 hr and then cultured with gp96-VSV19 complex for another 2 hr in presence or absence of the inhibitor. The cells were chromium labeled at the same time for 1 hr and then washed and used as targets against CD8<sup>+</sup>T cells specific for VSV8 in a 4 hr chromium release assay. Gp96-VSV19, lactacystin-untreated pulsed APCs were lyzed by VSV8-specific CD8<sup>+</sup> T cells (FIG. 10A). As observed previously for gp96 (Suto and Srivastava 1995, *supra*) and for hsp70 (Castellino *et al.*, 2000, *supra*), only a small proportion of pulsed APCs were lyzed by the APCs even at the highest E:T ratio tested (FIG. 10A). The APCs pulsed with VSV8 (through surface charging) were lyzed in a titratable and more significant degree, indicating that the APCs were entirely capable of being lyzed. The basis of the selective lyzability of APCs re-presenting HSP-chaperoned peptides is still unclear. However, and regardless of this observation, the lactacystin-treated, gp96-VSV19 pulsed APCs were not recognized by the VSV8-specific CD8<sup>+</sup> T cells and were not lyzed by them (FIG. 10A). Inhibition of proteasomal function thus inhibits the processing of peptides chaperoned by gp96 (FIG.10A). As other HSPs tested also use the same portal of entry (FIG. 9), it is assumed that inhibition of proteasome function interferes with processing of peptides chaperoned by them as well. The data recently reported by Castellino *et al.* for hsp70 are consistent with this inference.

*Re-representation of gp96-chaperoned peptides by MHC I of the APCs requires a functional TAP.* The requirement of TAP in re-representation of gp96 chaperoned peptides by APCs was tested. In a re-representation assay *in vitro*, gp96 purified from liver or the same gp96 complexed with VSV19 was pulsed on to primary cultures of peritoneal macrophages derived from TAP <sup>+/+</sup> or <sup>-/-</sup> mice. The pulsed APCs were used to stimulate CD8<sup>+</sup> T cell lines specific for K<sup>b</sup>/VSV8. After incubation for 20 hr, the culture supernatants were tested for release of IFN-γ as a marker for T cell stimulation (FIG.10B). It was observed that APCs from TAP<sup>+/+</sup> mice stimulated the CD8<sup>+</sup> T cells specifically when cultured in presence of gp96 complexed to VSV19 but APCs from TAP1<sup>-/-</sup> mice were unable to do so. This result indicates that gp96-chaperoned peptides must enter the endoplasmic reticulum through the TAP molecules, for being loaded on the MHC I molecules. As other HSPs tested also use the same portal of entry (FIG. 9), it is assumed that peptides chaperoned by other HSPs also require TAP for re-representation. Part of the data recently reported by Castellino *et al.* for hsp70 are consistent with this inference.

In studies *in vivo*, TAP1<sup>-/-</sup> (C57BL/6/SV129J) or wild type (C57BL/6) mice were immunized with the gp96-VSV19 complexes (50 μg of gp96 complexed with 50 μg of

VSV19), or VSV19 alone, or gp96 alone. Spleen cells of immunized mice were cultured with the VSV8 and tested for cytotoxic activity on <sup>51</sup>Cr labeled EL4 cells or EL4 cells pulsed with the VSV8 peptide as targets. Spleen cells of wild type (C57BL/6) mice immunized with gp96-peptide complex showed VSV8-specific CTL activity whereas splenocytes of TAP1 (-/-) mice immunized with gp96-peptide complex showed no cytotoxic activity (FIG.10C). It may be argued that the lack of CTL activity in TAP-/- mice is a result of the poor loading and stability of MHC I molecules in general, rather than because of a specific block in re-presentation. While this is possible, and is difficult to entirely refute, we are easily able to generate VSV8-specific CTLs in TAP-/- mice as in TAP+/+ mice by immunization with VSV8 peptide in incomplete Freund's adjuvant (data not shown). Sandberg et al. (1996) have reported similar data. In any case, the data from re-presentation assays in vitro using APCs from TAP+/+ and -/- mice (FIG. 10B) demonstrate the TAP requirement for re-presentation convincingly and without the complexity introduced by the experiment in vivo (FIG. 10C).

## 6.4 DISCUSSION

The  $\alpha$ 2M receptor, which is also designated CD91, was initially identified as a protein related to the low density lipoprotein (LDL) receptor Related Protein (LRP) (Strickland *et al.*, 1990, J. Biol. Chem.265:17401-17404; Kristensen *et al.*, 1990, FEBS Lett. 276:151-155). The protein consists of an ~420 kDa  $\alpha$  subunit, an 85 kDa  $\beta$  subunit and a 39 kDa tightly associated molecule (RAP). The  $\alpha$  and  $\beta$  subunits are encoded by a single transcript of ~15 Kb in size (Van Leuven *et al.*, 1993, Biochim. Biophys. Acta. 1173:71-74). The receptor has been shown to be present in cells of the monocytic lineage and in hepatocytes, fibroblasts and keratinocytes. CD91 has been shown previously to bind the activated form of the plasma glycoprotein  $\alpha$ 2M, which binds to and inhibits a wide variety of endoproteinases.  $\alpha$ 2M receptor also binds to other ligands such as transforming growth factor  $\beta$  (O'Connor-McCourt *et al.*, 1987, J. Biol. Chem. 262:14090-14099), platelet-derived growth factor (Huang *et al.*, 1984, Proc. Natl. Acad. Sci. U.S.A. 81:342-346), and fibroblast growth factor (Dennis *et al.*, 1989, J. Biol. Chem. 264:7210-7216).  $\alpha$ 2M is thus believed to regulate, and specifically diminish, the activities of its various ligands. Complexed with these various ligands,  $\alpha$ 2M binds  $\alpha$ 2M receptor on the cell surface and is internalized through receptor-mediated endocytosis. Uptake of  $\alpha$ 2M-complexed ligands has been assumed thus far to be the primary function of the  $\alpha$ 2M receptor, although a role for it in lipid metabolism is also assumed.  $\alpha$ 2M receptor ligands other than  $\alpha$ 2M, such as tissue-specific plasminogen activator-inhibitor complex (Orth *et al.*, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:7422-7426) and urokinase-PAI1 complex (Nykjaer *et al.*, 1992, J. Biol. Chem. 267:14543-14546), have been identified. These ligands attest to a role for  $\alpha$ 2M receptor in



clearing a range of extracellular, plasma products.

The studies reported here show that the heat shock proteins gp96, hsp90, hsp70, and calreticulin are additional ligands for the  $\alpha 2$ M receptor. The human gp96-coding gene has been mapped previously by us at chromosome 12 (q24.2→q24.3) (Maki *et al.*, 1993, Somatic  
5 Cell Mol. Gen. 19:73-81). It is of interest in this regard that the  $\alpha 2$ M receptor gene has been mapped to the same chromosome and at a not too distant location (q13→q14) (Hilliker *et al.* Genomics 13:472-474). Gp96 binds  $\alpha 2$ M receptor directly and not through other ligands such as  $\alpha 2$ M. Homogenous preparations of gp96, in solution, or cross-linked to a solid matrix, bind to the  $\alpha 2$ M receptor. Indeed, the major ligand for the  $\alpha 2$ M receptor,  $\alpha 2$ M,  
10 actually inhibits interaction of gp96 with  $\alpha 2$ M receptor, instead of promoting it, providing evidence that gp96 is a direct ligand for the  $\alpha 2$ M receptor. The 80 kDa protein shown to bind gp96 is clearly an amino terminal degradation product of the  $\alpha$  subunit of the  $\alpha 2$ M receptor. Degradation products of the  $\alpha 2$ M receptor in this size range have also been observed in previous studies (Jensen *et al.*, 1989, Biochem. Arch. 5:171-176), and may  
15 indicate the existence of a discrete ectodomain in the  $\alpha 2$ M receptor which may be particularly sensitive to proteolytic cleavage.

The studies shown here also indicate that the  $\alpha 2$ M receptor is also engaged by hsp90, hsp70 and calreticulin. This observation is surprising in light of the fact that hsp70, calreticulin and hsp90/gp96 have no obvious structural similarities with each other. In  
20 another context, HSPs have presented us with this dilemma before: many of the various HSPs have no obvious homologies with each other and yet they appear to bind many of the same peptides (Ishii *et al.*, 1999, J. Immunol. 162(3):1303-1309; Breloer *et al.*, 1998, Eur. J. Immunol. 28(3):1016-1021). It remains to be seen if grp170, which belongs to the extended hsp70 family and hsp110, which has no homology to any of the other HSPs, shall share the  
25 CD91 receptor. The multiple common properties of the HSPs which share the Fourth Paradigm (Srivastava P.K., 1994, Experientia 50(11-12):1054-1060), i.e. peptide-binding, interacting with APCs through a common receptor, ATP-binding and ATPase activity, strongly suggest that these molecules must share conformational similarities which are not obvious from their primary sequence. Crystallographic analyses of the HSPs have begun to  
30 reveal their structure (Zhu *et al.*, 1996, Science 272:1602-1614; Prodromou *et al.*, 1997, Cell 90:65-75; Stebbins *et al.*, 1997, Cell 89:239-250), and shall shed light on this question.

The observations that  $\alpha 2$  macroglobulin and anti-CD91 antibodies inhibit re-presentation by each of the four HSPs completely, indicate that CD91 is the only receptor for the 4 HSPs. Considering the increasingly obvious role which the HSPs play in innate  
35 (Basu *et al.*, 2000, Int. Immunol. 12(11):1539-1546) and adaptive immune response, this observation is somewhat counter-intuitive. However, the data on complete inhibition by two independent means (FIG.. 9) are quite compelling. We have noticed earlier, and we report

here, significant differences between hsp70 and hsp90/gp96 in their ability to compete for binding to gp96 receptors (Binder *et al.*, 2000, J. Immunol. 165:2582-2587). Another group has also observed similar differences between gp96 and hsp70 (Arnold-Schild *et al.*, 1999, 162:3757-3760). These differences are not inconsistent with our present report pointing to a single receptor for the 4 HSPs. They simply suggest that the various HSPs interact with a single receptor with widely differing affinities. Castellino *et al.* have recently demonstrated re-presentation of hsp70-chaperoned peptides by APCs through receptor-mediated uptake and have suggested the existence of receptors of different affinity classes for single HSPs. This argument is biologically cogent, but is not supported by our present data.

Once the HSP-peptide complex binds to the receptor, peptides chaperoned by the HSPs must enter the APC along with the HSP. The studies reported here address the downstream events solely with respect to gp96 in the assumption that if all HSPs enter through the same portal, the downstream events must be identical or similar for peptides chaperoned by each of them. Our observations suggest that the peptides go from the endosome to the cytosol, to the ER and then to the secretory pathway to be re-presented on the surface. The transit through the cytosol is established through the proteasome requirement as well as through the TAP requirement of re-presentation. There is no known mechanism for transit of molecules from vesicular to soluble compartment although precedents certainly exist (Chiang *et al.*, 1989, Science 246:382-385). Exploration of this pathway shall, without doubt, open a new window into our understanding of intracellular traffic of proteins. Castellino *et al.* have reported recently on the events as they occur downstream of receptor-mediated uptake of hsp70-peptide complexes by APCs (Castellino *et al.*, 2000, *supra*). Our observations with a different HSP (gp96) are entirely consistent with that version of events and buttress the notion that the same portal of entry is used by all the peptide-chaperoning HSPs for re-presentation.

As shown here, the heat shock protein- $\alpha$ 2M receptor interaction provides a new type of function for  $\alpha$ 2M receptor, a function of a sensor, not only of the extracellular environment with its previously known plasma-based ligands, but also a sensor of the intracellular milieu as well. HSPs such as gp96 are obligate intracellular molecules and are released into the extracellular milieu only under conditions of necrotic (but not apoptotic) cell death. Thus, the  $\alpha$ 2M receptor may act as a sensor for necrotic cell death (see FIG. 11), just as the scavenger receptor CD36 and the recently identified phosphatidyl serine-binding protein act as sensors of apoptotic cell death and receptors for apoptotic cells (Savill *et al.*, 1992, J. Clin. Invest.90:1513-1522; Fadok *et al.*, 2000, Nature 405:85-90). Interaction of the macrophages with the apoptotic cells leads to a down-regulation of the inflammatory cytokines such as TNF (Fadok *et al.*, 2000, *supra*), while gp96-APC interaction leads to re-presentation of gp96-chaperoned peptides by MHC I molecules of the APC, followed by

stimulation of antigen-specific T cells (Suto and Srivastava, 1995, *supra*) and, in addition, secretion of pro-inflammatory cytokines such as TNF, GM-CSF and IL-12. Interestingly,  $\alpha$ 2M, an independent ligand for the  $\alpha$ 2M receptor, inhibits representation of gp96-chaperoned peptides by macrophages. This observation suggests that re-presentation of  
5 gp96-chaperoned peptides can not occur physiologically in blood, but only within tissues as a result of localized necrotic cell death. This is consistent with the complete absence of gp96 or other HSPs in blood under all conditions tested. Together, these observations point towards a possible mechanism whereby the release of HSPs in the blood as a result of severe tissue injury and lysis will not lead to a systemic and lethal pro-inflammatory cytokine  
10 cascade.

It is possible, therefore, that the  $\alpha$ 2M receptor renders it possible for the APCs to sample (i) the extracellular milieu of the blood through  $\alpha$ 2M and other plasma ligands and (ii) the intracellular milieu of the tissues through HSPs, particularly of the gp96 family. The former permits APCs to implement their primordial phagocytic function, while the latter  
15 allows them to execute its innate and adaptive immunological functions. Viewed in another perspective, recognition of apoptotic cells by APCs through CD36 or phosphatidyl serine, leads to anti-inflammatory signals, while interaction of the APC with necrotic cells through  $\alpha$ 2M receptor leads to pro-inflammatory innate and adaptive immune responses (see Srivastava *et al.*, 1998, *Immunity* 8: 657-665).

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The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention.  
25 Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein, including patent applications, patents, and other  
30 publications, are incorporated by reference herein in their entireties for all purposes.

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